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STUDIES ON THE YEASTS SACCHAROMYCES CEREVISIAE AND SACCHAROMYCOPSIS LIPOLYTICA.

A Thesis submitted to the University of Warwick for the Degree of Doctor of Philosophy.

This research was mainly carried out in the Department of Molecular Sciences, University of Warwick. Some results were also obtained at the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa., U.S.A.. The Appendix contains a report of work done at the British Petroleum Research Centre, Sunbury-on-Thames, Middlesex.

M. D. Skipton.

November, 1974.

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ABBREVIATIONS.

ADP	Adenosine-5'-diphosphate.
ANS	1-Anilinonaphthalene-8-sulphonic acid.
ATP	Adenosine-5'-triphosphate.
ATPase	Adenosine-5'-triphosphatase.
BSA	Bovine serum albumin.
CAP	Chloramphenicol.
CCCP	Carbonyl cyanide-m-chloro-phenylhydrazone.
$CCCP^R$	Mutant having resistance to CCCP.
CF_o-F_1	Reconstituted mitochondrial membrane preparation, obtained by Kagawa and Racker, (1966), having oligomycin-sensitive ATPase activity.
CHX	Cycloheximide.
DCA	N, N'-Dichloroethyl-p-aminophenyl-acetic acid.
DCCD	N, N'-Dicyclohexyl-carbodiimide.
DCPIP	2, 6-Dichlorophenol-indophenol.
DDA^+	Dimethyl-dibenzyl-ammonia.
DNA	Deoxyribonucleic acid.
DNP	2, 4-Dinitrophenol.
EDTA	Ethylenediamine-tetra-acetic acid.
EPR	Electron paramagnetic resonance.
ER	Erythromycin.
F_1-ATPase	Mitochondrial protein fraction, first isolated by Pullman et al, (1960), having ATPase activity which is cold labile and oligomycin insensitive.
FCCP	p-Trifluoromethoxy-carbonyl cyanide-phenyl hydrazone.
mt-DNA	Mitochondrial deoxyribonucleic acid.
mt-RNA	Mitochondrial ribonucleic acid.
mt-mRNA	Mitochondrial messenger RNA.
mt-rRNA	Mitochondrial ribosomal RNA.
mt-tRNA	Mitochondrial transfer RNA.

NAD	Nicotinamide adenine dinucleotide.
NADP	Nicotinamide adenine dinucleotide phosphate.
NADH	Reduced NAD.
NADPH	Reduced NADP.
NAD(P)	Either NAD or NADP.
NAD(P)H	Either NADH or NADPH.
NPA	2-azido-4-nitrophenol.
OL	Oligomycin.
OL^R	Mutant having resistance to oligomycin.
OS-ATPase	Oligomycin-sensitive ATPase from bovine heart or S. cerevisiae mitochondria. (Tzagoloff et al, 1968a, b; Tzagoloff and Meagher, 1971).
OSCP	Oligomycin-sensitivity conferring protein.
p	To indicate whether Saccharomyces sp. has "petite" mutation or not; i.e. p^-="petite", p^+= normal.
Pi	Inorganic phosphate.
PMS	Phenazine methosulphate.
RNA	Ribonucleic acid.
mRNA	Messenger RNA.
rRNA	Ribosomal RNA.
tRNA	Transfer RNA.
SDS	Sodium dodecylsulphate.
SMP	Submitochondrial particle.
sp.	Species.
S-13	5-Chloro-3-t-butyl-2'-chloro-4'-nitro-salicylanilide.
SF-6847	3, 5-Di-tert-butyl-4-hydroxy-benxylidene malononitrile.
TBA	Tributylamine.
TET	Tri-ethyl tin.
TET^R	Mutant having resistance to tri-ethyl tin.
$(TET)_2SO_4$	Tri-ethyl tin sulphate.
TMPD	N, N, N', N'-tetramethyl-p-phenylenediamine.
TPB^-	Tetraphenylboron.
TTFB	4, 5, 6, 7-tetrachloro-2-trifluoromethyl-benzimidazole.
$TTFB^R$	Mutant having resistance to TTFB.
UNC	Uncoupler.
UNC^R	Mutant having resistance to uncoupler.
"1799"	1, 1, 5, 5-trifluoromethyl-1, 5-hydroxy-pentan-3-one.
"1799"^{R}	Mutant having resistance to "1799".

SUMMARY

The elucidation of the biochemistry of oxidative phosphorylation is a problem capable of no easy solution. Combined genetic and biochemical investigation is a more recent approach in this direction.

Mutants of the yeast Saccharomyces cerevisiae resistant to inhibitors or to uncouplers of oxidative phosphorylation have been isolated in this Laboratory. In part, this Thesis is an investigation into their further properties. Growth characteristics of selected strains are described together with differences in their cytochrome profiles. The phosphorylation capabilities of isolated mitochondria have also been assayed. The effects of the various agents on cellular and mitochondrial respiration are measured.

Resistance to uncouplers may be manifest at the mitochondrial level; but in any case this does not lead to a more efficient energy conservation process.

The metabolism of hydrocarbons by microorganisms is now becoming of greater importance. Saccharomycopsis lipolytica is a yeast which is able to grow on n-alkanes of medium chain length. Growth curves and cytochrome contents of S. lipolytica cultured on various substrates, including n-alkanes, are compared. Isolated mitochondria are also examined.

Biological membranes contain characteristic fatty acids. The fatty acid profiles of cells and mitochondria of S. cerevisiae and of S. lipolytica after growth on various substrates are illustrated. The kinetics of membrane bound respiratory enzymes are affected by these lipid constitutents as evidenced by Arrhenius plots.

CHAPTER 1. Introduction

Energy metabolism in biological systems has been the subject of intensive study and has seen the development of many important concepts (Kalckar, 1969). However the precise biochemistry of the energy conservation mechanisms present in oxidative phosphorylation and in photosynthesis still remains to be elucidated. In bacteria, as in all prokaryotes, these processes are localised in the cell membrane, but in eukaryotes these energy conservation reactions are associated with particular organelles within the cell, i.e. mitochondria and chloroplasts respectively.

Several hypotheses have been put forward to explain the phosphorylation of ADP linked to the operation of the mitochondrial respiratory chain. The "chemical intermediate" hypothesis (Slater, 1953) was virtually unchallenged until the "chemiosmotic" mechanism was formulated by Mitchell, (1961). In reply there have been a number of subsequent proposals based on variations of the "chemical intermediate" hypothesis (Chance et al, 1967). The "conformational" hypothesis (Boyer, 1965; Boyer et al, 1973) has also received further support (Hackenbrock, 1966; Harris et al, 1968; Watson et al, 1971). These hypotheses are equally applicable to photosynthetic phosphorylation and it is possible for any one to provide an interpretation of almost any experiment. A comparison of these various hypotheses is provided by Greville,(1969) and more specific treatments of oxidative phosphorylation and energy coupling in mitochondria are given by Lardy and Ferguson, (1969), van Dam and Meyer, (1971) and Harold, (1972). A discussion of photosynthetic phosphorylation in bacteria and in higher plants by Walker and Crofts, (1970) is also available.

It has been possible to study the characteristics of various energy dependent reactions of mitochondrial oxidative phosphorylation. These depend on the existence of a relatively intact coupling system in isolated mitochondria or submitochondrial particles. Useful information has also been obtained from measurements of partial reactions of oxidative phosphorylation and from examination of the respiratory chain and phosphorylation enzymes in uncoupled systems (Myers and Slater, 1957; Chance, 1972).

Another general approach has been to try to resolve the oxidative phosphorylation complex into its component parts, and then to reconstitute a functional system from its separate components. The respiratory chain has been broken down into "Complexes" by use of detergents to solubilise the inner mitochondrial membrane (Hatefi et al, 1962 a, b; Fowler et al, 1962; Kagawa, 1972). The mitochondrial ATPase complex has been the subject of separation and reconstitution experiments by Racker and co-workers (Kagawa, 1972). By using the appropriate respiratory chain enzyme complex, together with purified phospholipids and coupling factors it has been possible to obtain vesicles which catalyse an isotopic exchange reaction (Kagawa and Racker, 1971) and also to reconstitute oxidative phosphorylation at Site I (Ragan and Racker, 1973) and at SiteIII (Racker and Kandrach, 1973). However, the exact mechanism of energy transfer to effect ATP synthesis is still unknown.

The elucidation of metabolic pathways in cells has been facilitated by the use of defective mutants. Using bacteria, eg. Escherichia coli and to some extent lower eukaryotes eg. Saccharomyces sp., it has been possible to show the involvement of a specific enzyme in a metabolic route by genetic and biochemical analysis. An alternative to this method is to select for mutants which are resistant to inhibitors of specific enzymically catalysed reactions which may be involved in the pathway. This approach has proved very useful in studies of bacterial protein synthesis (Beneveniste and Davies, 1973; Haselkorn and Rothman-Denes, 1973).

Both these approaches have recently been applied to the study of energy conservation mechanisms. E. coli mutants defective in energy transduction have been characterised (Butlin et al, 1971; Gutnick et al, 1972; Butlin et al, 1973; Nieuwenhuis et al, 1973; Cox et al, 1974). Strains which are resistant to DCCD, an inhibitor of coupled phosphorylation, have also been isolated in E. coli (Nieuwenhius et al, 1973) and in Streptococcus faecalis (Baron and Abrams, 1971; Abrams et al, 1972).

The isolation of mutants affected in some component of the energy conservation apparatus is more complicated in the case of eukaryotes because both mitochondria and chloroplasts posses their own particular DNA, together with transcription and translation apparatus, which may be differentiated from

those of the nucleus and cytoplasm (Sager, 1972). Simple eukaryote organisms have been used to provide mutants with defects in their energy conservation systems. Strains of Chlamydomonas rheinhardii, which have an inability to carry out functional photosynthetic electron transport (Levine, 1969; Levine and Goodenough, 1970), have been obtained together with similar mutants defective in the terminal stages of ATP synthesis (Sato et al, 1971b). The "poky" and related strains of Neurospora crassa are unable to carry out oxidative phosphorylation (Mitchell et al, 1953; Lambowitz et al, 1972). These mutants characteristically involve cytoplasmic genetics (Sager, 1972).

E. coli and Saccharomyces sp. offer the most profitable systems for the twofold approach of genetic analysis coupled with biochemical experimentation. There is a large amount of information already available on the genetics of E. coli and isolation of the membrane ATPase has been reported (Bragg and Hou, 1972).

The classical system for the study of mutants lacking functional oxidative phosphorylation are the Saccharomyces yeasts. These possess "petite" strains which can arise by spontaneous mutation, and which were first characterised by Ephrussi and co-workers (Slonimski and Ephrussi, 1949; Ephrussi, 1953). A discussion of the detailed genetic properties of these strains is given by Sager, (1972). There are limitations on the usefulness of mutants in the study of bioenergetics, the most important being the difficulty of routinely isolating strains with defects in their energy conservation system(s) since such a mutation is almost certainly lethal. The second problem has been the experimental difficulties inherent in dissecting membrane bound enzyme complexes into their functional component parts. Saccharomyces yeasts have the capacity for growth on fermentable and non-fermentable carbon scources. This increases the possibility of isolating a mutant with a deficient oxidative phosphorylation system since it would grow slowly or not at all on the oxidisable substrate but relatively well on the fermentable substrate. The isolation of a yeast mutant with a primary defect in energy coupling to ATP synthesis would be significant since it would then be possible to correlate genetic observations with biochemical studies. The protein subunits of the ATPase complex of Saccharomyces cerevisiae mitochondria have been recently described (Tzagoloff et al, 1973) and similar studies on the cytochrome oxidase complex from this yeast have been made by Schatz and co-workers (Mason et al, 1972).

According to Nass et al, (1965) mitchondrial DNA is an integral part of probably all mitochondria. It is now evident that the establishment of complete, functional mitochondria in the yeast cell requires the cooperation of the mitochondrial and nuclear genetic systems, together with their corresponding transcription and protein synthesis apparatus. This process can be influenced by the external environment of the cell eg. type of carbon scource or availability of oxygen. The structure of the mitochondria in the cell is therefore determined by two sets of genetic information, each of which is capable of mutation to a greater or lesser extent.

Saccharomyces yeast have for some time provided the natural medium for the study of mitochondrial biogenesis. It is of interest to know the exact contribution of each genome to the protein content of the mitochondrion and also the sites of translation of the corresponding protein components. The mitochondrial DNA of animals is normally circular and 4.8-5.9 μ in length (Borst and Flavell, 1972) and that of unicellular organisms or plants is longer (15-30 μ). The mitochondrial DNA of Saccharomyces yeast is circular and 25 μ long (Hollenberg et al, 1970; Borst and Flavell, 1972). All the mt-DNA's that have been studied hybridise with their corresponding mt-rRNA's and mt-tRNA's. There is one gene for each of the mt-rRNA's in animal mitochondria and the same situation probably exists for unicellular eukaryotes. The mitochondrial genetic system does not therefore have multiple rRNA cistrons and yeast mutants which are resistant to antibiotics which specifically affect mitochondrial protein synthesis could equally well have mutation(s) in mt-rRNA rather than changes in the ribosomal proteins. The mitochondrial genetic and protein synthetic systems have more in common with those of bacteria than with the eukaryote nuclear apparatus and elucidation of the various properties of the two genetic systems in the yeast cell has been made possible by differential inhibition experiments. Labelling of mt-RNA in vivo is possible (Dawid, 1972b)and separation of mt-RNA synthesis from nuclear transcription has also been achieved (Dubin, 1972). RNA synthesis by mitochondria in vitro has also been recently demonstrated (Michaelis et al, 1972).

Mitochondrial ribosomes can be differentiated from their cytoplasmic counterparts, and correspondingly, mt-rRNA shows no similarities to cytoplasmic rRNA. In unicellular eukaryotes the mitochondrial ribosomes are generally 70-74 S in size and made up of 50 S and 30-40 S subunits (Borst, 1972).

The mitochondrial rRNA species are 21-24 S and 14-16S with a G+C content of about 26% in yeast (Grivell et al, 1971 a, b). Yeast mitochondrial ribosomes have been shown to be active in vitro using bacterial protein factors and an artificial template, eg. Poly U or R17 RNA, (Sala and Kuntzel, 1970).

It must be assumed that the mitochondrial system follows the universal code (Swanson, 1971). Experiments designed to isolate the tRNA species specific to mitochondria have shown that both the mt-DNA strands carry genetic information (Buck and Nass, 1969; Nass and Buck, 1970). However, the 4S fraction of mt-RNA, which would be expected to contain the mt-tRNA's covers a smaller than expected number of genes on mt-DNA (Reijinders and Borst, 1972). In order to increase the number of different mt-tRNA molecules to even the minimum demanded by the genetic code (approximately 60) it is necessary to consider the possibility that cytoplasmic tRNA molecules may be imported into the mitochondria. Initiation of protein synthesis on the bacterial ribosome is with N-formyl methionine plus its corresponding tRNA and the initiator in eukaryotic cytoplasmic translation systems is methionyl -tRNA (Lucas-Lennard and Lipmann, 1971). The existence of N-formyl methionyl tRNA which will hybridise with mt-DNA, has been shown in yeast mitochondria by Smith and Marcker, (1968), together with the corresponding synthetase enzymes (Halbriech and Rabinowitz, 1971).

Differential inhibition experiments with CAP and CHX have been used to indicate that the proteins making up the structure of the mitochondrion are from both the mitochondrial and cytoplasmic translation systems (Clark-Walker and Linnane, 1966 a, b). However, the observation that CAP inhibits incorporation of labelled amino-acids is not by itself sufficient evidence to prove that a particular protein is mitochondrially synthesised. The drug may also have other effects on cellular metabolism eg. respiration (Firkin and Linnane, 1968). Nevertheless from these and other lines of evidence such as derepression or anaerobic-aerobic transition experiments it is possible to estimate that approximately 10% of the mitochondrial proteins are mitochondrially synthesised (Nass, 1969). Isolation of these proteins has proved difficult since they are incorporated into the inner membrane and are therefore rather insoluble (Schatz, 1970). There is now no evidence for the "structural protein" postulated by Woodward and Munkres, (1966) as this preparation has been shown to consist of

a mixture of different proteins (Senior and MacLennan, 1970). However, the protein subunits of the oxidative phosphorylation complex must also fulfill a role as structural components of the inner mitochondrial membrane (Pullman and Schatz, 1967). It is significant that for establishment of energy coupling the mitochondrially synthesised components are necessary even though most of the complex consists of nuclear coded proteins.

Several mitochondrial proteins are nuclear coded and synthesised on cytoplasmic ribosomes (Schatz, 1970). Yeast cytochrome c is a clear example (Sherman et al, 1966), but this also applies to most of the proteins of the inner membrane (Henson et al, 1968; Schatz et al, 1972), all of the outer membrane proteins (Neupert et al, 1967; Beattie et al, 1967) and the protein constitutents of the mitochondrial matrix (Roodyn et al, 1962). Most of the protein components of the mitochondrial genetic system are also produced in this way (Davey et al, 1969; Wintersberger, 1970; Scragg, 1971a). There is evidence for mitochondrial coding coupled with mitochondrial synthesis of proteins from genetic analysis of drug resistant mutants. Certainly resistance to CAP and to ER is cytoplasmically determined (Bolotin et al, 1971; Saunders et al, 1971). Resistance at the ribosomal level to these agents would imply some change(s) in the overall structure of the ribosome (Grivell et al, 1971) and this is certainly the case with the bacterial system where drug resistance is due to modification of ribosomal proteins (Beneveniste and Davies, 1973). The situation with yeast is more complicated however since most mitochondrial ribosomal proteins are synthesised on cytoplasmic ribosomes (Lizardi and Luck, 1972). Dawid, (1972a) has postulated that at least in animals mt-DNA may only code for mt-tRNA's and mt-rRNA's while all the proteins are nuclear coded and mitochondrially synthesised.

In trying to identify the sites of coding and translation of mitochondrial proteins there is the possibility of isolation of mt-mRNA and hybridisation studies with mt-DNA. The other approach has been to try to obtain drug resistant strains with a mutation in the mitochondrial DNA and attempt to link this mutation with a corresponding change in a mitochondrially synthesised protein. It is not possible to use the well characterised "petite" mutation since this normally has too great a lesion in the mt-DNA, and in any case it has no functional protein synthetic system (Schatz et al, 1970). Antibiotics have been used to differentiate between the mitochondrial and cytoplasmic protein synthetic systems in yeast and also to pinpoint the site of transcription of mitochondrial components.

Sensitivity to inhibition by rifampicin and resistance towards α-amanitin are characteristic of the mitochondrial DNA-dependent RNA polymerase in eukaryotes (Scragg, 1971 b). Lack of effect of rifampicin on intact mitochondria is due to the lack of permeation of the drug across the inner membrane (Fukamachi et al, 1972). Ethidium bromide does however, inhibit transcription of mitochondrial DNA in intact cells with virtually no detectable effect on nuclear RNA synthesis (Zylber et al, 1969) while Actinomycin D gives precisely the same result for transcription of nuclear DNA (Knight, 1969; Kroon and Arendzen, 1972). These observations must be viewed with caution because of permeability, detoxification and specificity effects together with differences in precursor pools which affect incorporation experiments.

The mitochondrial and nuclear genetic systems can be said to be interdependent but differential inhibition experiments at the translational level give no direct information on the site of transcription and vice versa. In support of the hypothesis of Dawid, that mt-DNA has a more regulatory function, Barath and Kuntzel, (1972) have provided evidence that most or all of the mitochondrial proteins in Neurospora crassa are nuclear coded and that cytoplasmic translation is controlled by repressors which are mitochondrially synthesised. However other workers have shown that some proteins fulfilling a structural role are mitochondrially synthesised and incorporated into the inner membrane (Wenner and Neupert, 1972). Mitochondrial protein synthesis must be functional to enable synthesis of cytochromes b, c_1 and aa_3 in mammalian cells (Kroon and De Vries, 1971) and in yeast it is possible to show that parts of the cytochrome oxidase and ATP synthetase complexes are mitochondrially translated (Mason et al, 1972; Tzagoloff et al, 1973).

In order to fix the sites of transcription and of translation of mitochondrial proteins in yeast several approaches have been tried. These have included "petite" mutants and CAP or CHX inhibited cells. The other main approach has been to define the characteristics of formation of functional mitochondria on transition from anaerobic conditions to an aerobic environment (Schatz, 1970; Watson et al, 1971). The cytology of the anaerobically grown cells is particularly influenced by the lipid composition of the growth medium (Morpurgo et al, 1964; Tustanoff and Bartley, 1964; Wallace et al, 1968) and also by catabolite repression (Linnane, 1965). Plattner et al, (1971) have shown that on aeration functional mitochondria are formed from promitochondria, but there is at present some confusion in the literature as to the exact characteristics of promitochondria from anaerobic, glucose-repressed, lipid-depleted cells. This may be due to technical difficulties in visualising the structures

for the electron microscope (Wallace et al, 1968; Damsky et al, 1969; Plattner and Schatz, 1969). Watson et al, (1971) provide evidence that the two types of mitochondrial precursors in anaerobic, glucose repressed cells, plus or minus lipid, are morphologically distinct. This is in contrast to the results of Schatz and co-workers (Criddle and Schatz, 1969; Paultauf and Schatz, 1969). Lipid depleted mitochondrial precursors apparently lack a protein synthesising system and this has led to speculation on the role of the inner mitochondrial membrane in protein synthesis (Dixon et al, 1971). Schatz et al, (1970) have shown that promitochondria from anaerobically grown, glucose repressed, lipid supplemented yeast do carry out CAP sensitive protein synthesis. This may be related to the fact that similar promitochondria catalyse energy transfer reactions in the absence of a respiratory chain (Groot et al, 1971).

Evidence has therefore accumulated which indicates that some mitochondrial proteins, which are incorporated into the inner membrane, are synthesised on mitochondrial ribosomes and that these proteins are essential for establishment of energy transfer reactions in functional mitochondria. Several mutants of S. cerevisiae at both the nuclear and cytoplasmic levels, have been obtained which are defective in some component of mitochondrial electron transport or coupled phosphorylation and these are discussed in Chapter 2 of this Thesis. Any single gene mutation which directly affects a protein component involved in the primary coupling of electron transport to ATP synthesis would yield important information which would complement the biochemical studies already made on mitochondria from yeast and other species.

Genetic analysis of the oxidative phosphorylation process in S. cerevisiae is closely related to the problem of mitochondrial biogenesis. Drug or antibiotic resistant mutants were first utilised in studying protein synthesis by yeast mitochondria (Linnane et al, 1968 a, b). On this basis a variety of inhibitors of mitochondrial electron transport or coupled phosphorylation have been used to obtain specifically resistant mutants of S. cerevisiae and other yeasts (Griffiths, 1972). Such mutants may provide information on the process of energy transfer in oxidative phosphorylation. Second, they may also give data on the biogenesis of yeast mitochondria with a possible application to the problem of the identity of the sites of coding and synthesis of mitochondrial proteins. Resistance by point mutation to an inhibitor or uncoupler having a well defined site of action in the oxidative phosphorylation process would be expected to be localised in only those protein components affected by the drug and therefore relatively specific. Studies on

cross resistance patterns may also give some information on the sites of action of these agents on membrane bound energy conservation systems (Houghton et al, 1974). Analysis of genetics would localise the mutation as either nuclear or cytoplasmic and biochemical investigation at the mitochondrial level could provide more data on the resistance phenomenon. Separation and purification of the protein subunits of the various enzyme complexes making up the complete oxidative phosphorylation system might lead to identification of a protein subunit with an amino acid change which could be correlated with a mutation in the mitochondrial DNA.

Yeast mutants have been obtained which are resistant to several types of inhibitors of electron transport or coupled phosphorylation. Antimycin resistant mutants of Candida utilis have been obtained by Butow and Zeydel, (1968) and Grimmelikhuijen and Slater, (1973). Nuclear mutants of Kluyveromyces lactis, resistant to alkyl guanidines, have been described by Brunner et al, (1973). Mutants resistant to oligomycin have been obtained and some have been characterised as nuclear mutations on single genes (Parker et al, 1968; Stuart, 1970; Goffeau et al, 1972). Cytoplasmically inherited oligomycin resistant strains of Saccharomyces cerevisiae have been obtained in our Department by Avner and Griffiths, (1970) and have been identified with single mutations on mitochondrial DNA (Avner and Griffiths, 1973 a, b). Similar series of cytoplasmically inherited, oligomycin resistant mutants have also been obtained by other workers (Wakabayashi and Gunge, 1970; Wakabayashi and Kamai, 1973; Mitchell et al, 1973). Other inhibitors of oxidative phosphorylation have also been used to select for specific yeast mutants eg. tri-alkyl tins (Lancashire and Griffiths, 1971) and bongkrekic acid (Lauquin et al, 1973; Perkins et al, 1973). Several types of mutants of S. cerevisiae resistant to uncouplers of oxidative phosphorylation or to ionophorous antibiotics have also been isolated in our Department (Griffiths et al, 1972; Griffiths, 1972), and characterised genetically.

In part this Thesis is an attempt at defining the characteristics of representative uncoupler or TET resistant strains of S. cerevisiae; together with a more detailed study of the biochemistry of the resistance phenomenon at the cellular and mitochondrial levels.

The latter Chapters in this Thesis describe changes in the energy conservation apparatus which occur when the yeast Saccharomycopsis lipolytica is grown on various substrates including n-alkanes. Measurements of these phenotypic variations are complementary to the above experiments with mutants of S. cerevisiae.

CHAPTER 2. Growth Characteristics and Cytochrome Contents of Representative Tri-ethyl Tin or Uncoupler Resistant Mutants of Saccharomyces cerevisiae.

INTRODUCTION.

Mutants of Saccharomyces cerevisiae resistant to inhibitors or to uncouplers of oxidative phosphorylation have been isolated in our Laboratory (Griffiths, 1972; Griffiths et al, 1972). The growth parameters of the wild type strain on both fermentable and non-fermentable substrates have been determined and compared with those of selected mutants. The rates of oxygen uptake of the cells during batch culture on ethanol have also been measured. There exists the possibility that mutants resistant to uncouplers may have a more efficient oxidative phosphorylation system than the wild type. This may result in higher growth yields and/or higher growth rates. The growth yields in batch culture have therefore been estimated according to the method of Kormancikova et al, (1969).

The cytochrome content of yeast cells can be affected by the genetic characteristics and also by the external environment. The phenomenon of glucose repression has been documented by Jayaraman et al, (1966) and promitochondria from anaerobic Saccharomyces sp. grown on glucose have been characterised by Schatz and co-workers (Plattner et al, 1971; Mason et al, 1972) and by Watson et al, (1971). Respiratory enzymes have been shown to be induced by oxygen (Ephrussi and Slonimski, 1950; Somlo and Fukuhara, 1965) and aeration - deaeration experiments confirm these observations (Luzikov et al, 1971). Involvement of the cytoplasmic genetic system in the biogenesis of mitochondrial components in S. cerevisiae has been accepted since the "petite" mutation was shown to be inherited as such (Ephrussi et al, 1949; Slonimski and Ephrussi, 1949). The isolation of mitochondrial DNA (Nass and Nass, 1963; Schatz et al, 1964) suggested a possible site for this cytoplasmic factor (Sherman, 1963). This has subsequently been

shown to be the case (Mounoulou et al, 1966; Mehrota and Mahler, 1968). "Petite" mutants do not grow on non-fermentable substrates and lack cytochromes aa_3, b and c_1. This pheno-type can also arise by nuclear mutation(s). Nuclear mutants having a variety of deficiencies in energy conservation, including a partial or complete lack of one or more cytochromes have been isolated and characterised (Sherman, 1964; Sherman and Slonimski, 1964; Reilly and Sherman, 1965; Sherman, 1967). Strains having "abnormal energy metabolism" have also been isolated (Parker and Mattoon, 1969).

The "petite" mutation is a gross change in the mt-DNA resulting in the complete loss of all cytochromes except cytochrome c. It is conceivable that more limited mutation(s) in the mt-DNA may give rise to more limited changes in the cytochrome profile. Some of the specific drug or uncoupler resistant mutants isolated in our Laboratory (Griffiths, 1972) also show more widespread effects on the properties of the inner mitochondrial membrane. Measurements of the cytochrome contents of cells and mitochondria of S. cerevisiae and selected TET or uncoupler resistant mutants have been made at room temperature and at $77^{o}K$.

Some of this work was carried out at the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa., U.S.A..

METHODS AND MATERIALS.

Triethyl-tin and Uncoupler Resistant Mutants of S. cerevisiae.

Mutants of S. cerevisiae, strain D22 (ad_2, α, p^+) were obtained in our Laboratory by treatment of wild type cells with ultra-violet light and selected by plating experiments using various concentrations of inhibitor or uncoupler (Griffiths, 1972). They have been grouped according to their genetics and this classification is shown in Table 2.1. All of the Class 1 mutants have nuclear genetics while the Class 2 mutants are cytoplasmic in origin. It should be noted that it is difficult to classify the $TTFB^R$ mutants on this basis; however similar results were also obtained with $CCCP^R$ mutants (P. R. Avner, personal communication). Strains selected for resistance to the uncoupler "1799" are by contrast relatively straightforward to classify. This may indicate some fundamental difference between uncouplers like DNP, CCCP or TTFB, and "1799". The levels of cross resistance of particular mutants to other inhibitors or uncouplers of oxidative phosphorylation or to antibiotics affecting mitochondrial protein synthesis have also been determined by plating experiments. These results complement the genetic studies and are summarised in Table 2.2. In every case the Class 1, nuclear mutants, are resistant to virtually every inhibitor of mitochondrial function, including protein synthesis inhibitors. The Class 2 strains are normally only resistant to the particular agent used for their selection, except that in the case of the $TTFB^R$ mutants resistance to CCCP, but not to "1799", is also expressed. The Class 3 mutants that are illustrated have the same genetic characteristics as those in Class 2 but retain cross resistance to "1799" only. This is also evidence for a difference between "1799" and the other uncouplers used. There may also be differences in resistance towards "acidic" uncouplers such as CCCP, and "basic" ones like S-13 but these have not been tested for. The Class 2 "$1799"^R$ mutants also retain some resistance to other uncouplers and also to TET which under certain conditions can uncouple electron transport from phosphorylation.

Lists of representative strains together with their cross resistance profiles are shown in Tables 2.3, 2.4 and 2.5 for the TET^R, $TTFB^R$ and $"1799"^R$ mutants respectively.

TABLE 2.1.

Genetic characteristics of various classes of TET or uncoupler resistant mutants of S. cerevisiae.

Criteria for Cytoplasmic Inheritance	TET^R		$TTFB^R$		"1799" ^{R}	
	Class 1	Class 2	Class 1	Class 2	Class 1	Class 2
Characteristic should show mitotic segregation in 'Rp^+ x Sp^+ diploids.	All colonies mixed.	Yes	All colonies mixed.	Yes	All colonies mixed.	Yes
Only R zygotes should be produced in Rp^+ x Sp^-	All colonies mixed.	Yes	Experiments not done		All colonies mixed.	Yes
p^- strains from R haploid no longer carry R allele.	No loss of resistance	Yes			No loss of resistance	Yes
Meiotic products of Rp^+ x Sp^+ should show 4:0 segregation.	2:2 segregation	Yes	Mutation to $TTFB^R$ makes spores non-viable, therefore tetrad analysis is impossible. Only 4% of all isolated spores actually grow and all are resistant.		2:2 segregation	Yes

Resistance or Sensitivity to an inhibitor or uncoupler is denoted by R or S respectively.

p^+ = wild type, p^- = "petite".

TABLE 2.2

Cross-resistance characteristics of inhibitor or uncoupler resistant mutants of S. cerevisiae.

Type	Class	Levels of Resistance (x wild type)				
		TET	Oligomycin	"1799"	TTFB	CCCP
TET^R	1	20	>20	8-12	4	-
	2	20	1	< 2	1	-
	3	20	1	8	1	-
$TTFB^R$	1	> 20	> 20	>10	6 - 8	6
	2	1	1	1	6 - 8	6
	3	1	1	8	6 - 8	10
1799^R	1	20	20	20	4	2
	2	10	1	20	2	2

TABLE 2.3

Resistance characteristics of TET resistant mutants of S. cerevisiae.

Class	Strain	Tolerance of strain to inhibitor or uncoupler (μg/ml)			
		TET	Oligomycin	"1799"	"TTFB"
	[2]D22	0.5	0.5	2.5	5
1	D22-EC7	10	>10	60	30
	D22-EC11	10	1.25	40	20
	D22-EC16	10	> 10	>60	>40
	D22-EC19	10	1.25	40	20
	D22-EC24	10	> 10	40	>40
2	D22-EB7	10	0.5	10	10
	D22-EC1	5	0.5	10-20	10
	D22-EC9	5	0.5	5	10
	D22-EC10	5	0.5	20	10
	D22-EC23	5	0.5	40	10
3	D22-EB8	10	0.5	40	10
	D22-EB16	5	0.5	40	10
	D22-EC2	10	0.5	40	10

[1]This Table is reproduced from Lancashire and Griffiths (1971).

[2]S. cerevisiae, strain D22 (ad_2, α, p^+) is the wild type strain.

TABLE 2.4

Resistance characteristics of TTFB resistant mutants of S. cerevisiae.[1]

Class	Strain	Tolerance of strain to inhibitor or uncoupler (μg/ml).				
		TET	Oligomycin	"1799"	TTFB	CCCP
	D22	0.5	0.5	2.5	5	1
1	D22-DB17	>10	>10	>40	30	4
	D22-DCS3	>10	>10	>40	30	6
2	D22-DB9	0.5	0.5	5	30	6
	D22-DC2	0.5	0.5	5	40	6
	D22-DC9	0.5	0.5	5	30	6
	D22-DCS12	0.5	0.5	5	60	6
3	D22-DC5	0.5	0.5	20	60	10
	D22-DCS9	0.5	0.5	20	30	10
	D22-DCS10	0.5	0.5	20	40	8
	[2]D22-DCS11	0.5	0.5	20	60	10

[1] This Table is constructed from data provided by Dr. W. E. Lancashire.

[2] D22-DCS11 reverts to wild type characteristics at 20°C.

TABLE 2.5

Resistance characteristics of "1799" resistant mutants of S. cerevisiae.

Class	Strain	Tolerance of strain to inhibitor or uncoupler (μ g/ml)				
		TET	Oligomycin	"1799"	TTFB	CCCP
	D22	0.5	0.5	2.5	5	1
1	D22-CB6	5	10	50	10	2
	D22-CB9	5	5	50	10	2
	D22-CB12	10	10	50	30	4
	D22-CB16	10	10	50	20	2
	D22-CB20	10	10	50	20	2
2	D22-CB1	10	0.5	50	10	2
	D22-CB2	2.5	0.5	40	10	2
	D22-CB7	10	0.5	50	10	2
	D22-CB10	5	0.5	50	10	2
	D22-CB19	10	0.5	50	10	2

' This Table is constructed from data supplied by Dr. W. E. Lancashire.

In conclusion it may be assumed that the Class 2 and Class 3 $TTFB^R$ mutants have so far been shown to be cytoplasmic but not necessarily mitochondrial in origin. In contrast the "1799"^{R}, Class 2 strains have mitochondrial genetics. The mutation to TET^R in Class 2 mutants, although mitochondrial, is not on the same mt-DNA as the other accepted markers eg. OL^1 and OL^2 (Avner and Griffiths, 1973a, b; W. E. Lancashire, personal communication).

All strains were obtained from Dr. W. E. Lancashire and maintained on plates [1.0% (w/v) yeast extract, 2.0% (w/v) peptone, 2.0% (w/v) agar and 2.0% (w/v) glucose] at 5 ^{o}C and sub-cultured every month.

Growth Curves on Ethanol or on Glucose.

The yeast were grown in a 500 ml conical flask containing 50 ml medium. This consisted of, per litre of distilled water : 0.5% (w/v) yeast extract; 1.0 g $(NH_4)_2SO_4$; 0.875 g KH_2PO_4; 0.125 g K_2HPO_4; 0.5 g $MgSO_4.7H_2O$; 0.1 g NaCl; 1.0g $CaCl_2.2H_2O$ added after autoclaving (Wickerham, 1946) and 0.1g adenine sulphate. The pH was adjusted to 5.5. The carbon scource was either glucose (0.4% w/v) or ethanol (0.5% v/v). The cultures were grown in the dark (Ninnemann et al, 1970) in a Gallenkamp rotary incubator at 30 ^{o}C and at 200-250 revs. per. min. A 1.0% (v/v) inoculum of a starter culture grown from a loop inoculum under the same conditions for 24-30 hr. was used. The flasks were equipped with a side arm which enabled direct measurements of the turbidity of the culture to be made using an EEL colorimeter (Evans Electroselenium Ltd., Halstead, Herts, U.K..). A calibration curve to relate the EEL absorbance reading to the dry weight concentration of yeast cells present in the culture was also made.

Growth and Respiratory Activity of S. cerevisiae on Ethanol.

The cultures were grown in a 2 ltr. conical flask, baffled for efficient aeration, containing 500 ml of medium. This was made up of 1.0% (w/v) yeast extract; mineral salts (Wickerham, 1946); and 0.1g adenine sulphate per litre of distilled water. The substrate was ethanol (0.5% v/v). This medium was buffered at pH 5.0 using 50 mM potassium phthalate. A 1.0% (v/v) inoculum from a starter culture grown for 24-30 hr. under the same conditions was used. The cultures were incubated in the dark (Ninnemann et al, 1970) at

30°C and shaken at 200-250 revs. per. min. on a Mark 5 shaker (L. H. Engineering Co., Stoke Poges, U.K.). Aliquots were periodically withdrawn by sterile pipette for the determination of the dry weight cell concentration of the culture. For assay of respiration a sample was also removed from the culture and spun down in a bench centrifuge and the cells washed and resuspended in 50 mM potassium phthalate, pH 5·0. The concentration of the cells was measured by dry weight assay. The oxygen uptake was followed at 30°C using a Rank electrode (Rank Bros., Bottisham, Cambridge, U.K.). The assay mixture contained:

1.9 ml 50 mM potassium phthalate, pH 5.0
20 µl absolute alcohol
0.1 ml yeast suspension (1.0-3.0 mg dry weight)

The initial oxygen concentration was estimated from Chance and Williams, (1967).

Molar Growth Yields of S. cerevisiae.

These were estimated in aerobic batch culture according to the method of Kormancikova et al, (1969). The yeast were grown in 500 ml conical flasks containing 50 ml of medium. This consisted of, in distilled water, 1.0% (w/v) yeast extract; 2.0% (w/v) casein hydrolysate (acid) and adenine sulphate (0.1 g/ltr). This medium was buffered to pH 5.2 using 50 mM potassium phthalate. The carbon source, added after autoclaving, was either glucose or ethanol in various concentrations. The cultures were grown in the dark in a Gallenkamp rotary incubator at 30°C and at 200-250 revs/min. A 1.0% (v/v) inoculum from a starter culture grown for 24-30 hrs. on 0.5% (v/v) ethanol was used. The flasks were equipped with side arms and growth was followed by reading the turbidity of the culture in an EEL colorimeter. When this measurement indicated that growth had ceased the culture was withdrawn from the incubator and the cell dry weight concentration assayed.

Growth of S. cerevisiae for Measurement of Cytochrome Content.

Cultures were grown in 2ltr conical flasks in the dark at 30°C on a Mark 5 shaker at 200-250 revs./min. The flasks were baffled for efficient aeration and contained 400 ml of medium. This consisted of, per litre of distilled water, 0.5% (w/v) yeast extract, mineral salts (Wickerham, 1946) and 0.1 g adenine sulphate. The pH was adjusted to 5.5 and the carbon

scource was ethanol at either 0.5% (v/v) or 1.0% (v/v) as indicated in the text. The inoculum was a 1.0% (v/v) sample of a starter culture previously grown on 0.5% (v/v) ethanol.

S. cerevisiae was also grown at 30°C in New Brunswick Fermenters (New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A.). In our Department, 10 ltr cultures were used and at the Johnson Foundation (University of Pennsylvania, Philadelphia, Pa., U.S.A.) 5 ltr cultures. In both cases the composition of the medium was as described above. Tri-butyl citrate at a concentration of 0.1-0.2 ml/ltr was used as antifoaming agent and the inoculum was a 1.0% (v/v) sample of a previously grown starter culture on 0.5% (v/v) ethanol. Stirring speed was always 500 r.p.m. and compressed air was bubbled through the culture at 1 ltr air/min/ltr medium.

Preparation of Mitochondria (Braun shaker).

Fermenter grown S. cerevisiae cells were harvested by centrifugation using an MSE "Mistral" instrument (6x 1ltr head, 2000 g, 10 min). This and all subsequent centrifugations were done at 0 - 5°C. The cells were washed with distilled water and resuspended in buffer (cell wet weight : buffer volume = 1 : 1). The buffer contained: 0.5 M Sorbitol
1 mM EDTA (disodium salt)
20 mM Tris-HCl, pH 8.0
0.2% (w/v) Bovine Serum Albumin (Fraction 5)

The cells were homogenised using a Braun shaker (B. Braun Apparatebau, Melsungen, Germany) with glass beads (0.45 - 0.5 mm diameter) at speed 2 (4000 r.p.m.) for 15 sec. The relative proportions were 1 part (by volume) cell suspension to 1 part (by weight) glass beads. The homogenate was centrifuged, using a Sorvall RC2-B centrifuge, at 1000g for 15 mins, and the supernatant again spun down at 1500g for 15 mins. This supernatant was then centrifuged at 2000g for 15 mins. and the pellet again discarded. The mitochondria were sedimented from the supernatant at 19000g for 40 mins. and suspended in buffer to a concentration of approximately 15 mg protein per ml. The buffer consisted of 0.25 M Sorbitol
1 mM EDTA (disodium salt)
20 mM Tris-HCl, pH 7.5.

The mitochondria could be further purified on a discontinuous sucrose gradient containing 70%, 50%, 30% and 20% (w/v) sucrose together with 1 mM EDTA (disodium salt) and 20 mM Tris -HCl, pH 7.5. Gradients were run on a Beckman L-2 ultracentrifuge using the SW 25.1 rotor at 23,000 r.p.m. for 2.5 hr. The mitochondrial band was collected and suspended in 0.25 M Sorbitol buffer (above) to a protein concentration of about 15 mg/ml.

Cytochrome Spectra at Room Temperature.

Cells were washed in distilled water and resuspended in buffer containing 60% (w/v) sorbitol, 50 mM potassium phosphate pH 7.0, at approximately 1.5 gm wet weight in 6 ml (15-25 mg dry weight per ml). The spectra were recorded on a Unicam SP 1800 spectrophotometer in 1 cm light path cells (final volume 2.5 - 3.0 ml) using the turbid solution facility with a slit width of 0.6 mm. The sample cell was reduced with a few grains of dithionite and the reference cell was oxidised with 10 μl of 100 vol. hydrogen peroxide. Difference spectra of mitochondria were recorded by the same method. The mitochondria were suspended in buffer, containing 0.25 M sorbitol, 1 mM EDTA, 20 mM Tris-HCl pH 7.5, to a concentration of about 4 mg protein per ml. and the reference cell was oxidised using 4 μl of 100 vol. hydrogen peroxide.

The difference spectra were analysed using the following wavelength pairs and extinction coefficients : Cytochrome c 550 - 540 nm, e_{mM} = 19 (Wilson and Epel, 1968); cytochrome b 560-540 nm, e_{mM} = 22 (Wilson and Epel, 1968); cytochrome aa_3 605 - 630 nm, e_{mM} = 24 (van Gelder, 1966).

Some cytochrome spectra of S. cerevisiae cells were measured at the Johnson Research Foundation (University of Pennsylvania, Philadelphia, Pa. U.S.A.), using a purpose built split beam instrument of their own design and manufacture.

Cytochrome Spectra at Liquid Nitrogen Temperature.

These were all measured at the Johnson Research Foundation. Aliquots were taken from the room temperature samples and placed in the sample and reference sides of a 3 mm light path perspex cell which was surrounded by copper cooling fins. The apparatus was then suspended in a vacuum flask containing liquid nitrogen, and the yeast suspension frozen. Transparent

windows were available in the flask so that light could pass through both cuvettes. Dry air was blown over these windows on the outside to avoid condensation problems. A description of the instrument used has been given by Chance, (1957).

Dry Weight and Protein Estimations.

Dry weights of intact cells were measured by filtration on to Whatman GF/C glass fibre filter paper (2.4 cm diameter) and dried in an oven at 110-120^{o}C to constant weight for 2 - 3 days. Protein estimations were by the method of Lowry et al, (1951).

Materials.

Yeast extract was obtained from Difco (Difco Laboratories Inc., Detroit, Michigan, U.S.A.) and peptone, casein hydrolysate (acid) and agar from Oxoid (Oxoid Ltd., London, U.K.). Adenine sulphate was purchased from B.D.H. (B.D.H. Chemicals Ltd., Poole, Dorset, U.K.) or from Sigma (Sigma (London) Chemical Co., Kingston-upon-Thames, U.K.). Bovine serumalbumin (fraction 5) was also obtained from Sigma. Tri-butyl citrate was a gift from British Petroleum (B.P. Research Centre, Sunbury-on-Thames, Middx., U.K.). All other chemicals were of Analytical Reagent grade where necessary.

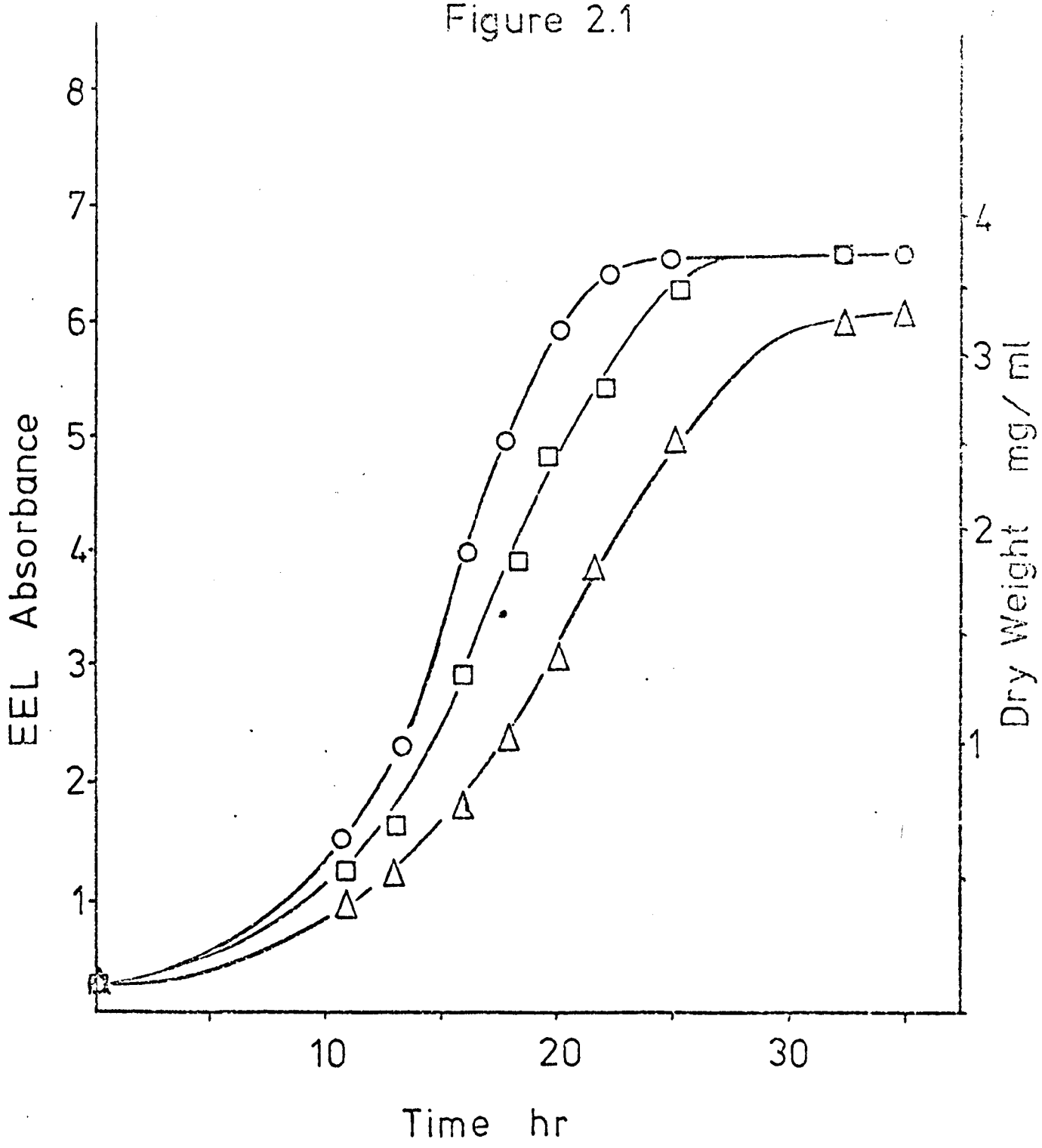
RESULTS

Growth of S. cerevisiae on Ethanol.

In Figure 2.1 the growth of S. cerevisiae, strain D22 (ad_2^-, p^+) on ethanol is compared with D22-DCS12 and D22-DCS9; $TTFB^R$ mutants of Class 2 and Class 3 respectively. Under these conditions the growth of these $TTFB^R$ mutants was defective. Establishment of the logarithmic phase of growth took longer, and in either case the maximum growth rate was less than that of the wild type, the strain D22-DCS9 having the longest generation time. Both D22 and D22-DCS 12 reached the same cell concentration at stationary phase (3.7 mg dry weight per ml). However, D22 took 25 hrs. but D22-DCS12 needed 28 hrs. to reach this point. For D22-DCS9 stationary phase occurred at 3.2 mg dry weight per ml after 34 hr. The growth characteristics of S. cerevisiae, D22 on ethanol are also compared with those of $TTFB^R$ mutants D22-DC9, D22-DC5 and D22-DCS11, (Figure 2.2). The strain, D22-DC9 is a Class 2 mutant while the others are members of Class 3. D22-DCS11 is a temperature sensitive strain losing resistance to TTFB at 20°C. The growth curves of the mutants D22-DC5 and D22-DC9 were comparable with that of D22-DCS9 in almost all respects. D22-DCS11 was the most defective of these mutants in terms of growth on ethanol. The lag phase of this strain was the longest and the maximum growth rate the slowest. Stationary phase was established at a cell concentration of 2.4 mg dry weight per ml after 35 hr growth in batch culture. In contrast to these $TTFB^R$ mutants, the "1799"^{R} strains D22- CB9 (Class 1) and D22-CB19 (Class 2), had growth curves under these conditions which were identical with that of the wild type (these results are not shown). Similarly, that of the TET^R mutant D22-EC2 (Class 3) was the same as the D22 wild type (Figure 2.3), but the Class 2 TET^R mutant, D22-EC1, did not match this; the maximum growth rate was lower and the stationary phase was reached after 30 hr at a cell concentration of 3.4 mg dry weight per ml. All these results were correspondingly the same when 1.0% (v/v) ethanol was used as substrate.

In order to provide some explanation for the differences in growth characteristics on ethanol, together with some information on the operation and devlopment of the respiratory chain, the rate of oxygen uptake of selected strains was measured throughout growth in batch culture. Figure 2.4 shows the

Figure 2.1



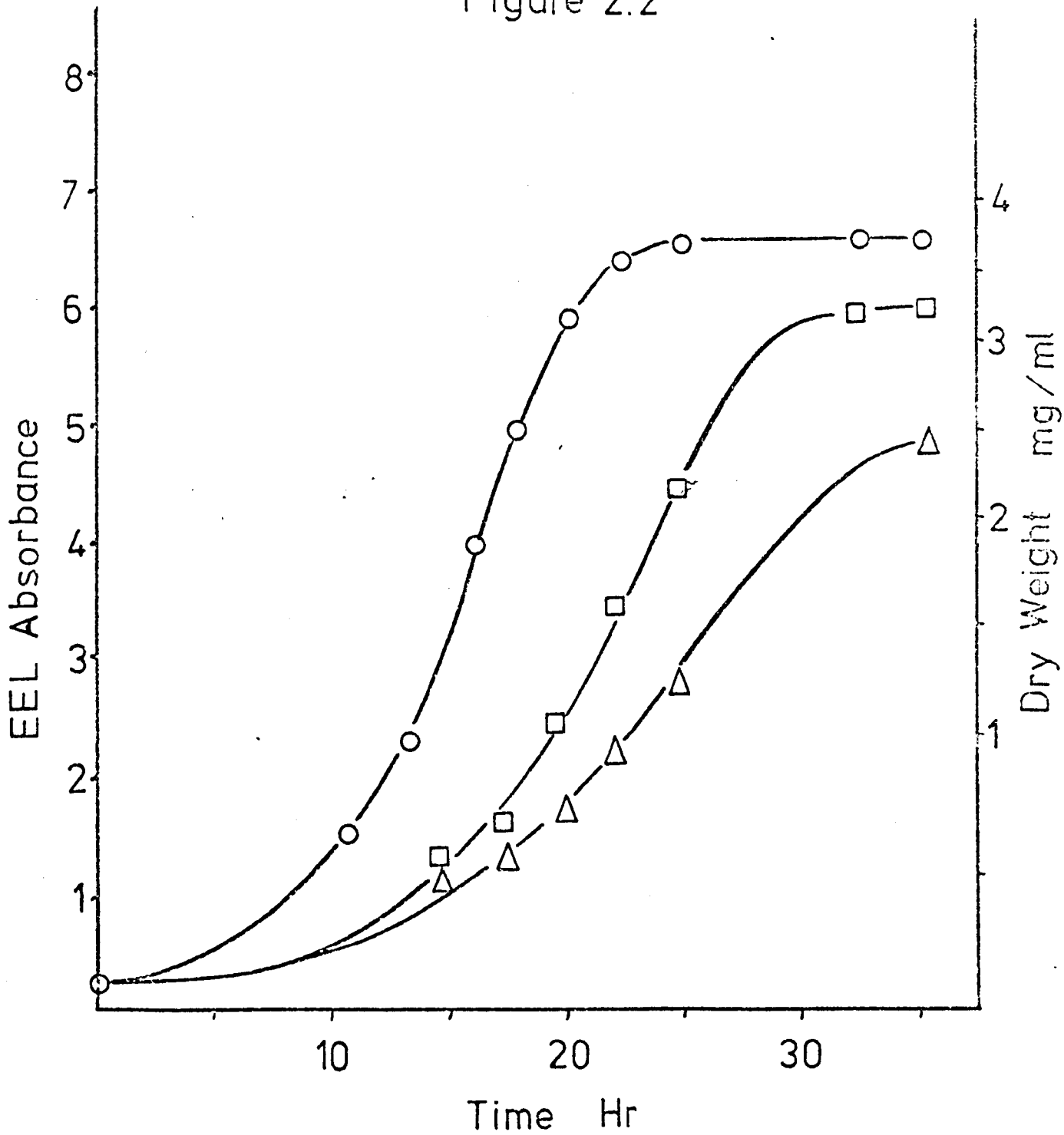
Growth curves of S. cerevisiae in shake flask culture on 0.5% (v/v) ethanol.

O - O D22

□ - □ D22 - DCS 12

Δ - Δ D22 - DCS9

Figure 2.2



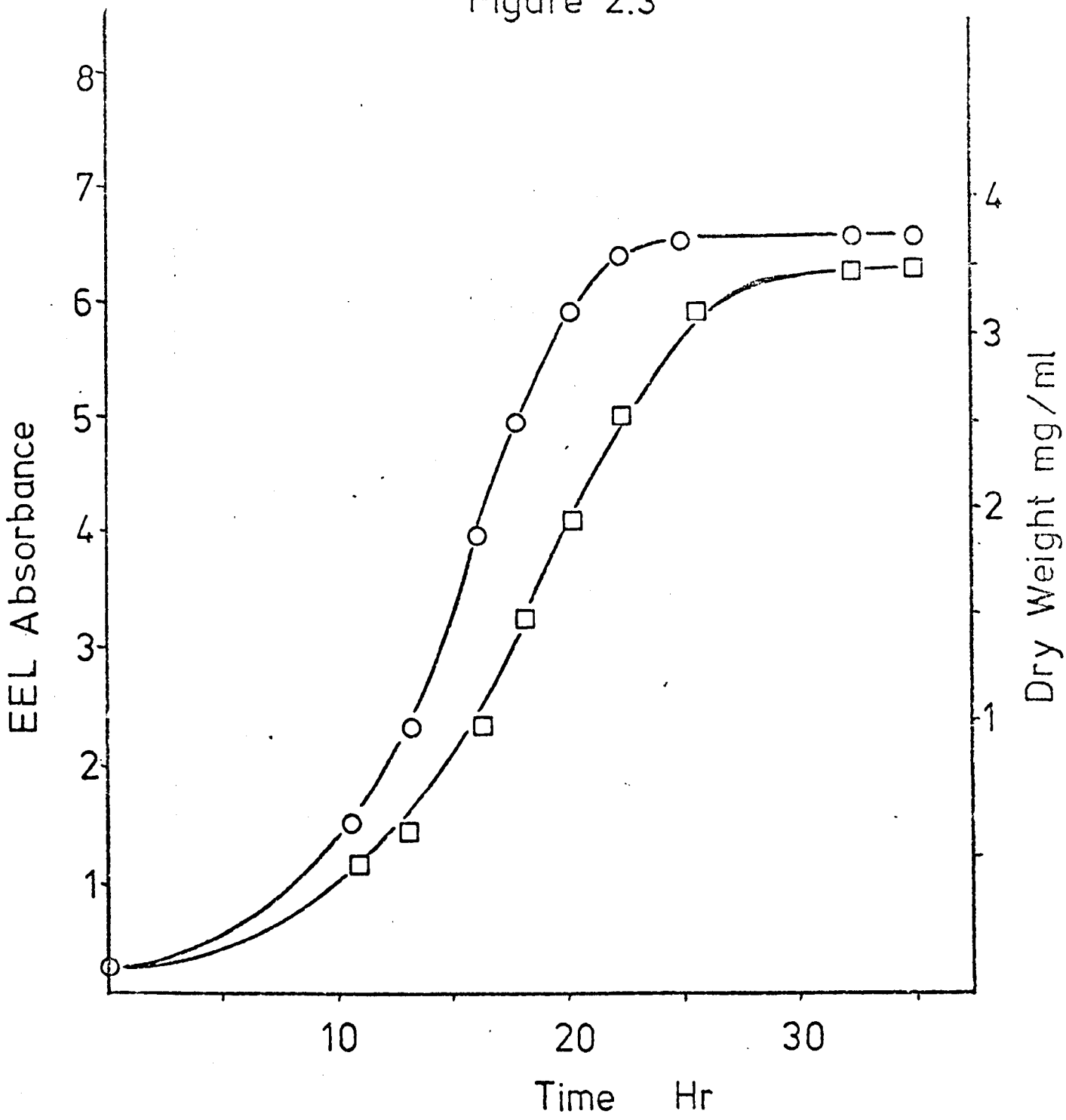
Growth curves of S. cerevisiae in shake flask culture on 0.5% (v/v) ethanol.

O - O D22

□ - □ D22 - DC5; D22 - DC9

Δ - Δ D22-DCS11

Figure 2.3

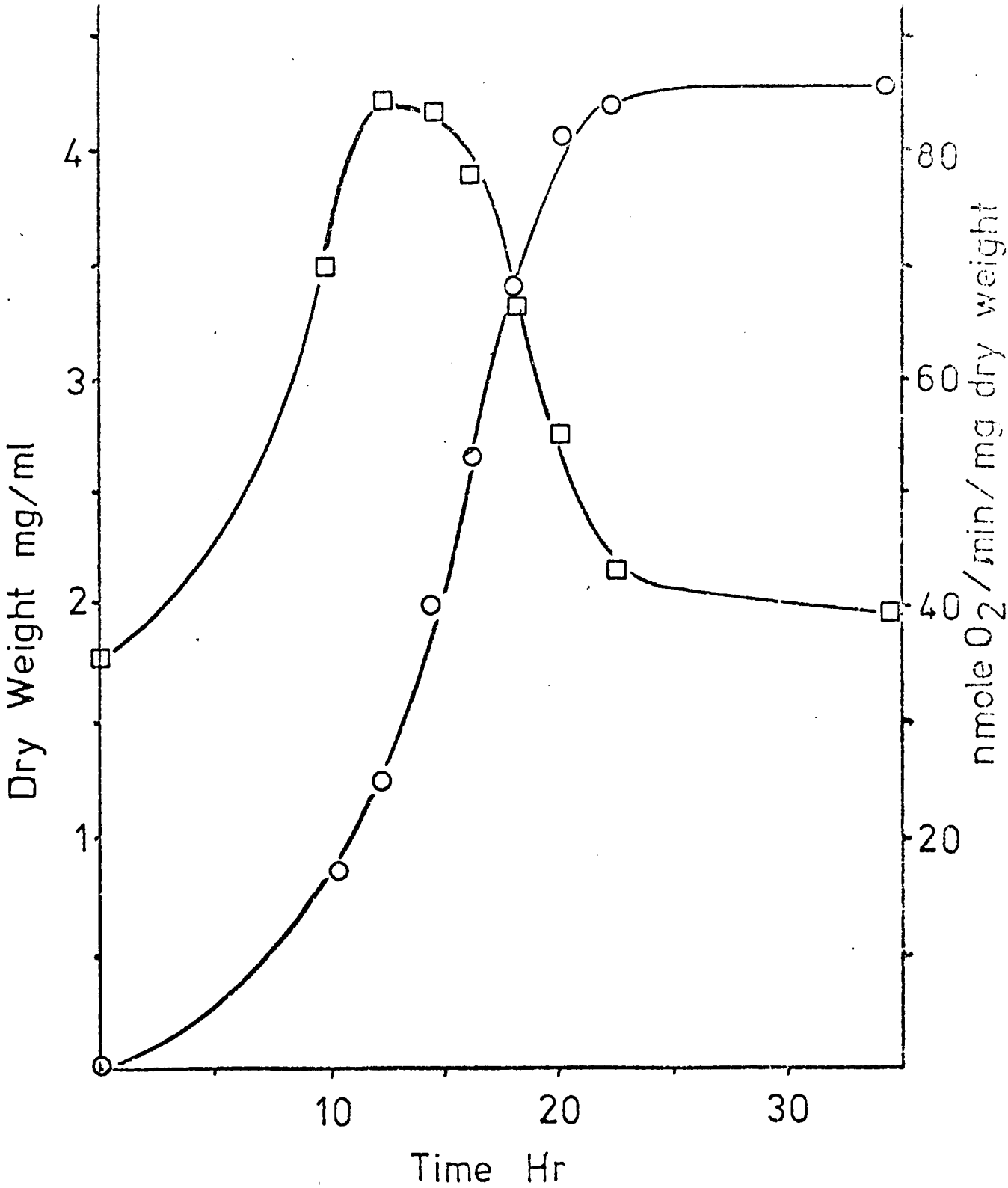


Growth curves of S. cerevisiae in shake flask culture on 0.5% (v/v) ethanol.

O - O D22, D22 - EC2

□ - □ D22- EC1

Figure 2.4

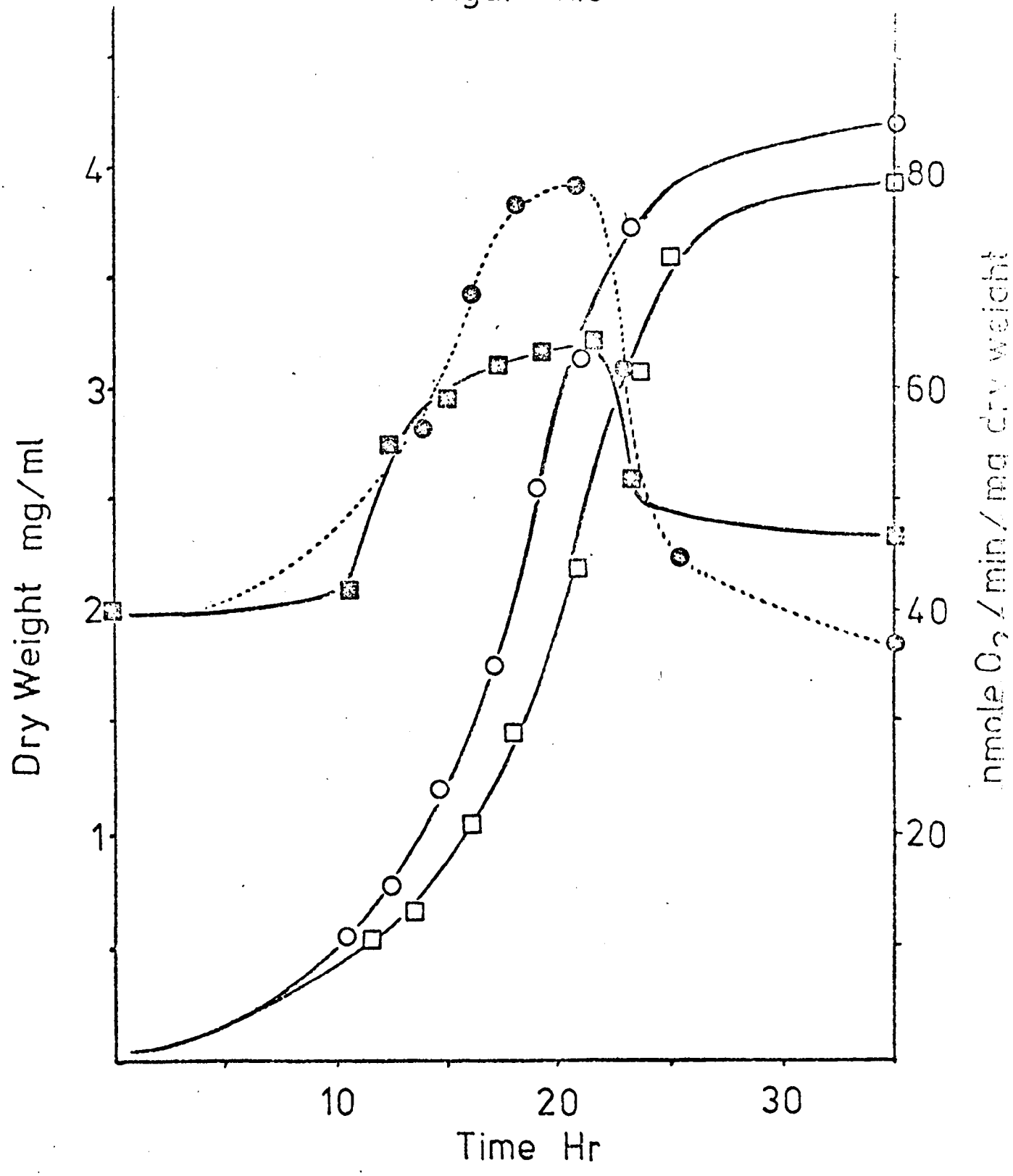


Growth curve and oxygen uptake of S. cerevisiae, strain D22 in shake flask culture on 0.5% (v/v) ethanol

O - O Dry weight mg/ml

□ - □ nmole 0_2/min/mg dry weight.

Figure 2.5



Growth curves and oxygen uptakes of uncoupler resistant mutants of S. cerevisiae in shake flask culture on 0.5% (v/v) ethanol.

D22 - DCS12	O - O	Dry weight mg/ml.
	● - ●	nmole 0_2/min/mg dry weight.
D22 - DCS9	□ - □	Dry weight mg/ml
	■ - ■	nmole 0_2/min/mg dry weight.

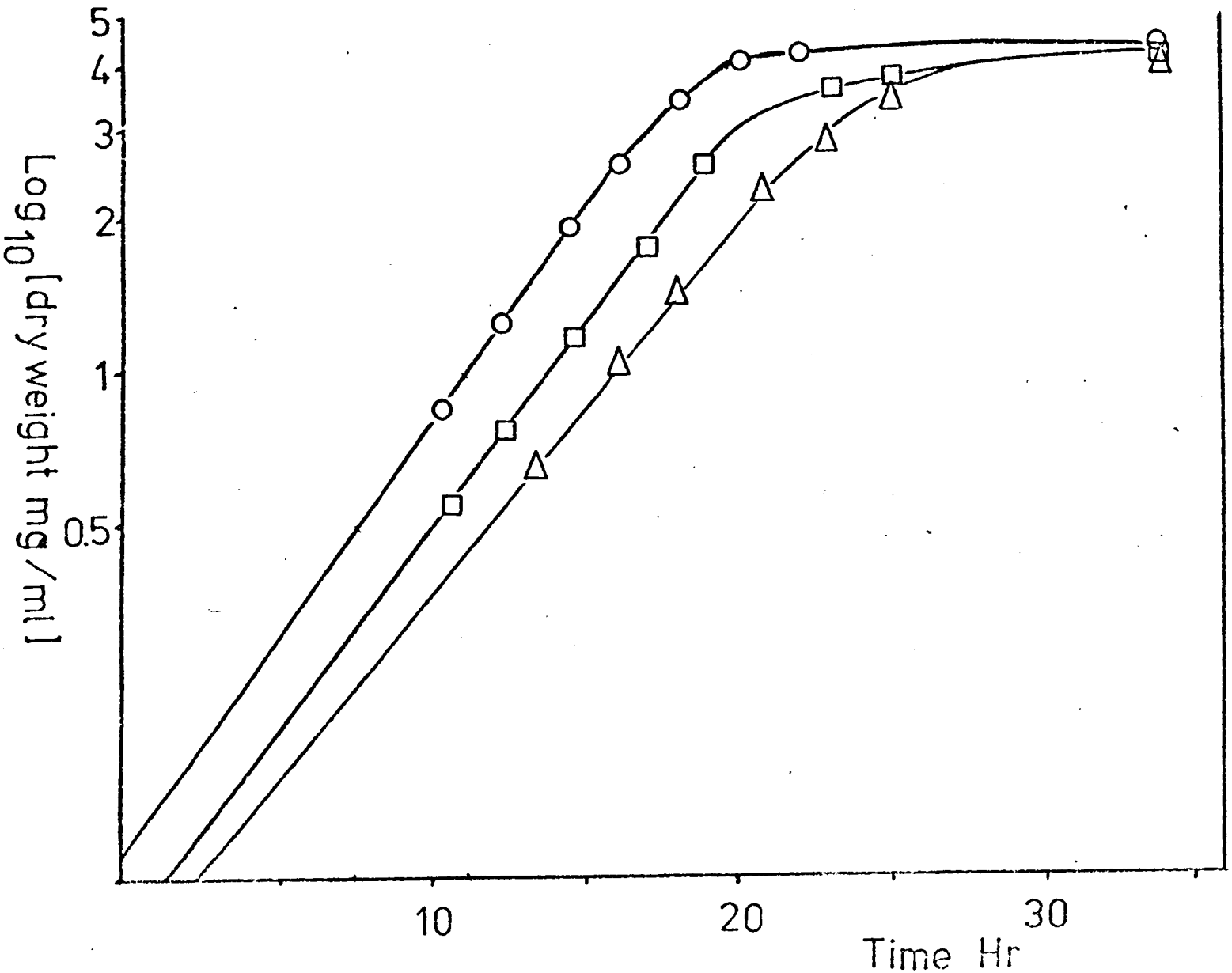


Figure 2.6

Logarithmic plots of the growth of S. cerevisiae in shake flask culture on 0.5% (v/v) ethanol.

O - O D22; D22-CB19.

□ - □ D22 - DCS 12.

Δ - Δ D22 - DCS 9.

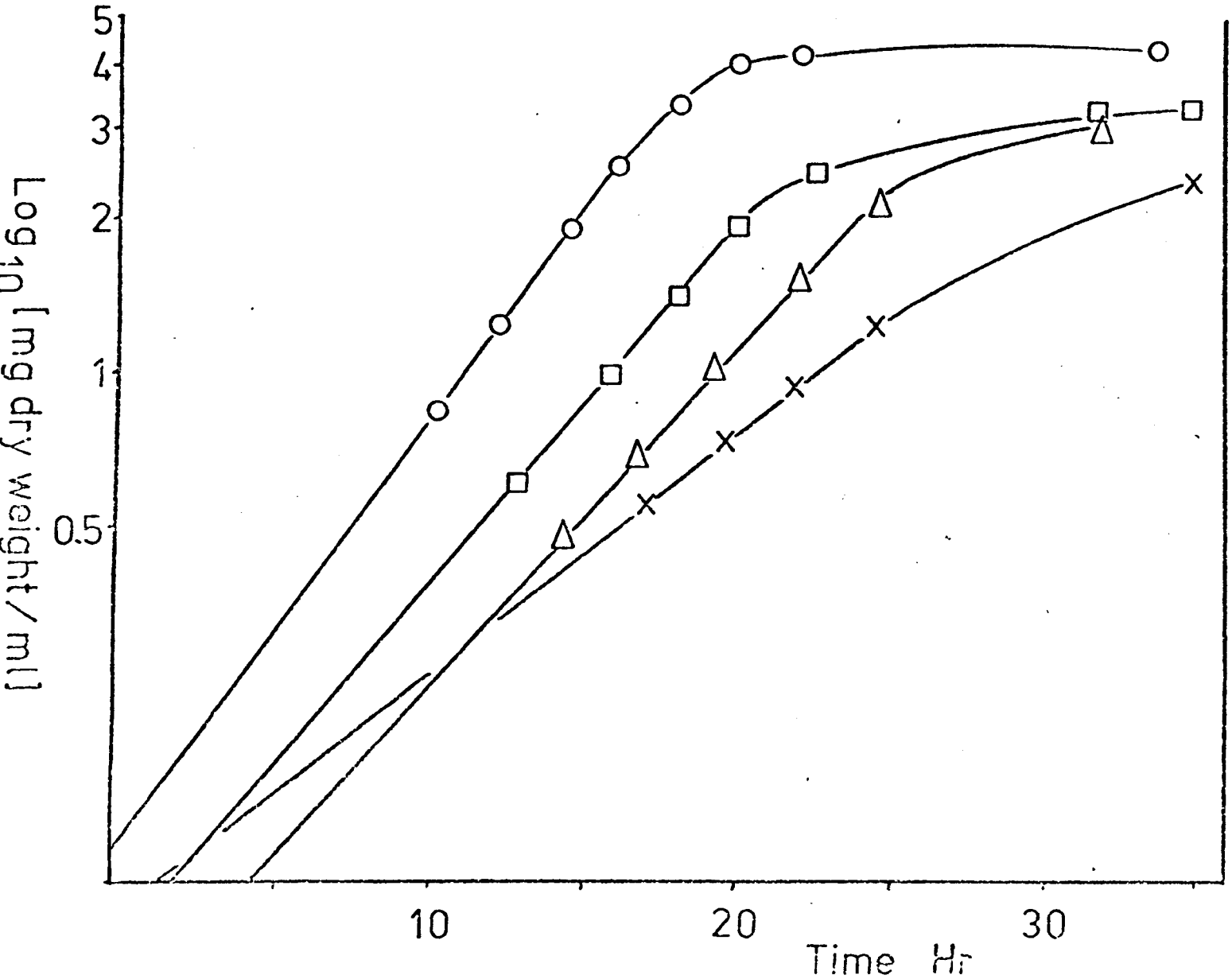


Figure 2.7

Logarithmic plots of the growth of S. cerevisiae in shake flask culture on 0.5% (v/v) ethanol

O - O D22; D22 - EC2.

□ - □ D22 - EC1.

Δ - Δ D22 - DC5; D22 - DC9.

X - X D22 - DCS11.

TABLE 2.6

Growth characteristics of S. cerevisiae on ethanol.

Strain	Type	Class	Generation Time hr.	Max respiration rate nmole 0_2/min/mg protein
D22	wild type	-	3.5	84
D22-DCS12	$TTFB^R$	2	3.8	78
D22-DC9	$TTFB^R$	2	4.6	N.T.'
D22-DC5	$TTFB^R$	3	4.6	N.T.
D22-DCS9	$TTFB^R$	3	4.0	64
D22-DCS11	$TTFB^R$	3	6.4	N.T.
D22-CB9	"1799"^{R}	1	3.5	N.T.
D22-CB19	"1799"^{R}	2	3.5	86
D22-EC1	TET^R	2	4.2	N.T.
D22-EC2	TET^R	3	3.5	87

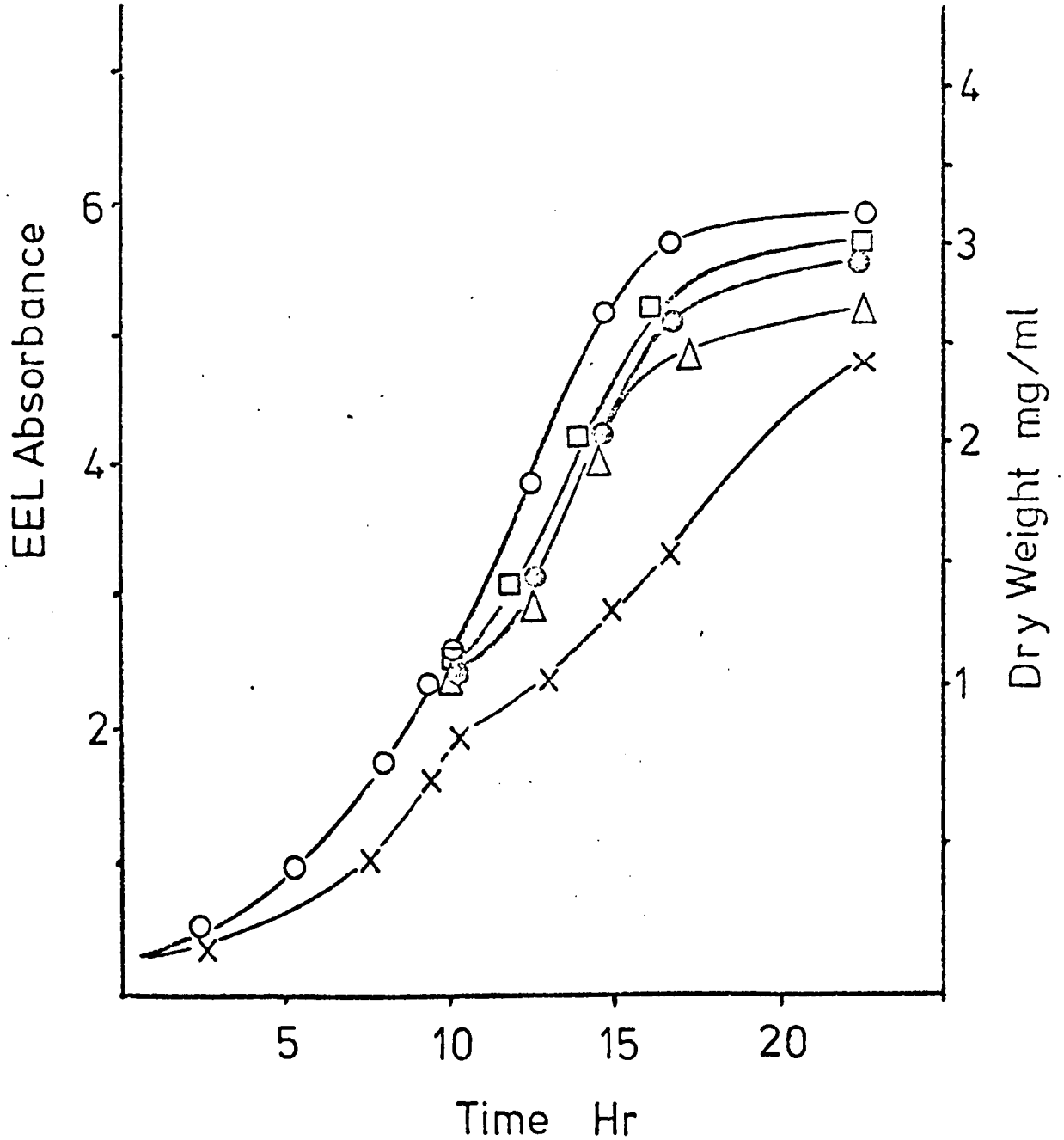
' N.T. = Not Tested.

increase in cell concentration together with the development of respiratory activity for the D22, wild type, strain. A maximum value of 84 nmoles 0_2 per min per mg dry weight was reached in the middle of the logarithmic phase of growth. This rate of oxygen uptake decreased to about 40 nmoles 0_2 per min per mg dry weight in lag and stationary phases. Figure 2.5 illustrates the results with the TTFB^R mutants D22-DCS12 and D22-DCS9. It is noticeable that neither of the mutants achieved the same maximal respiratory rate as the wild type strain. These were 78 and 64 nmoles 0_2 per min per mg dry weight for D22-DCS12 and D22-DCS9 respectively. This maximal rate of respiration was achieved near the end of the logarithmic phase of growth for both mutant strains. The rates of respiration of the "1799"^{R} mutant, D22-CB19 and the TET^R mutant, D22-EC2 were also measured (results not shown) and the values were in both cases of the same order as that of the wild type.

These data correlate with the maximal observed growth rates and show that defects in respiration or in coupled phosphorylation may be associated with variations in growth parameters of the mutants. The slightly higher growth yields found in Figures 2.4 and 2.5 when compared with Figures 2.1 - 2.3 were due to the slightly different composition of growth medium that was used.

The growth rate of each strain on ethanol can be estimated from Figures 2.6 and 2.7 (see Table 2.6). These are semi-logarithmic plots of Figures 2.1 - 2.5. These graphs also provide a means to check that all strains grow logarithmically. The mutants fall into three distinct groups. Firstly those with generation times of the same order as the wild type ie. D22-DCS12, D22-EC2, D22-CB9 and D22-CB19; these also had a maximal rate of oxygen uptake in the same range as that of the wild type. In some cases the rate of respiration of the mutant was slightly higher than that of the wild type (Table 2.1) but these variations were small and probably due to errors in measurement. The second group includes D22-DC5, D22-DC9, D22-DCS9 and D22-EC1 where the generation times are 20-25% longer than that of the wild type. The maximal rate of respiration observed in one example of this group, D22-DCS9, was also about 20% less than that of the wild type. Only one example of the third group was encountered, this is the TTFB^R mutant, D22-DCS11. The generation time was almost double that of the wild type, but oxygen uptake studies were not done.

Figure 2.8



Growth curves of S. cerevisiae in shake flask culture on 0.4% (w/v) glucose.

O - O D22.

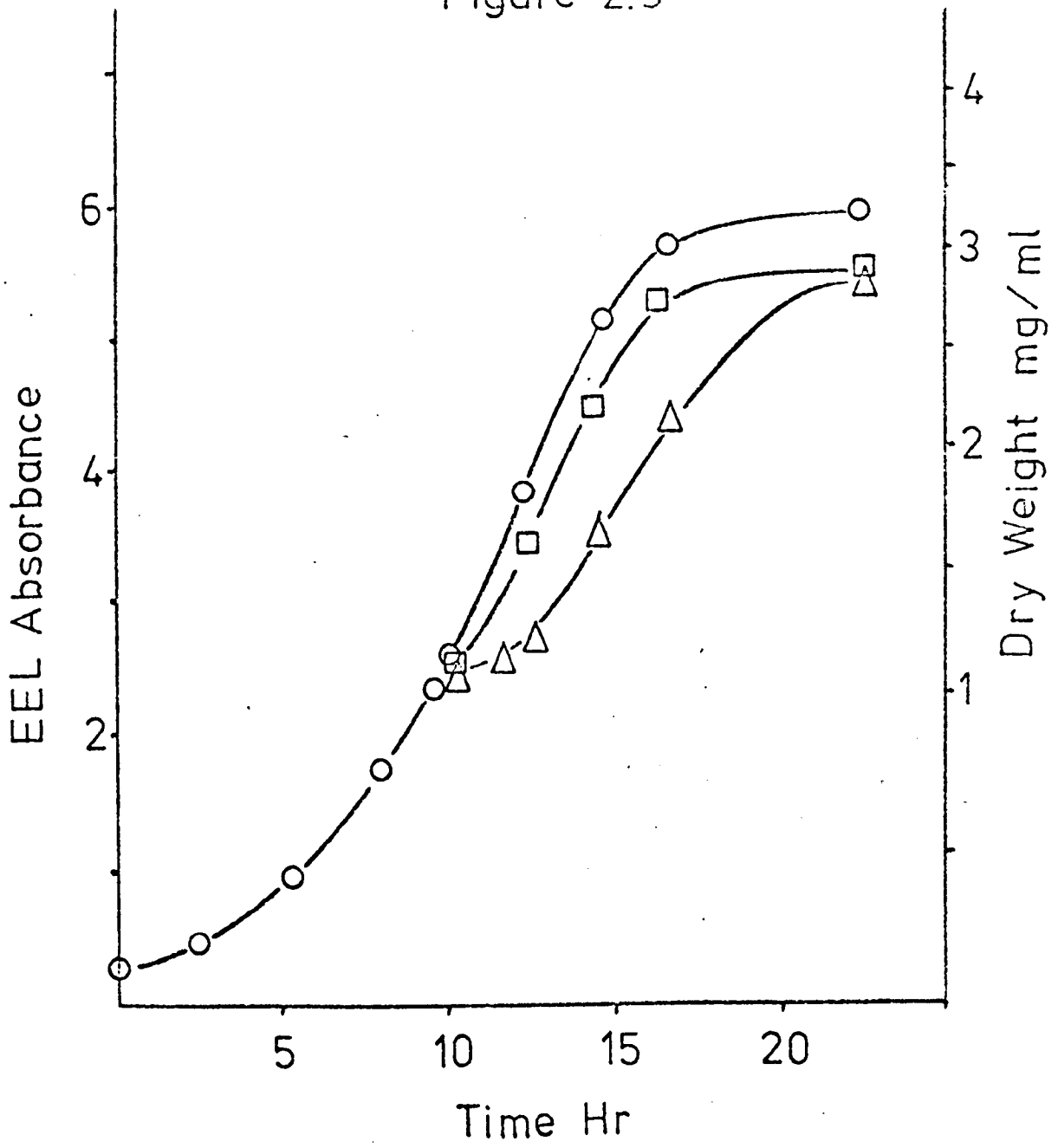
□ - □ D22 - DCS 12.

● - ● D22 - DCS 9.

Δ - Δ D22 - DC 5, D22 - DC 9.

X - X D22 - DCS 11.

Figure 2.9



Growth curves of S. cerevisiae in shake flask culture on 0.4% (w/v) glucose.

O - O D22, D22 - EC2.

□ - □ D22 - CB9, D22 - CB19.

Δ - Δ D22 - EC 1.

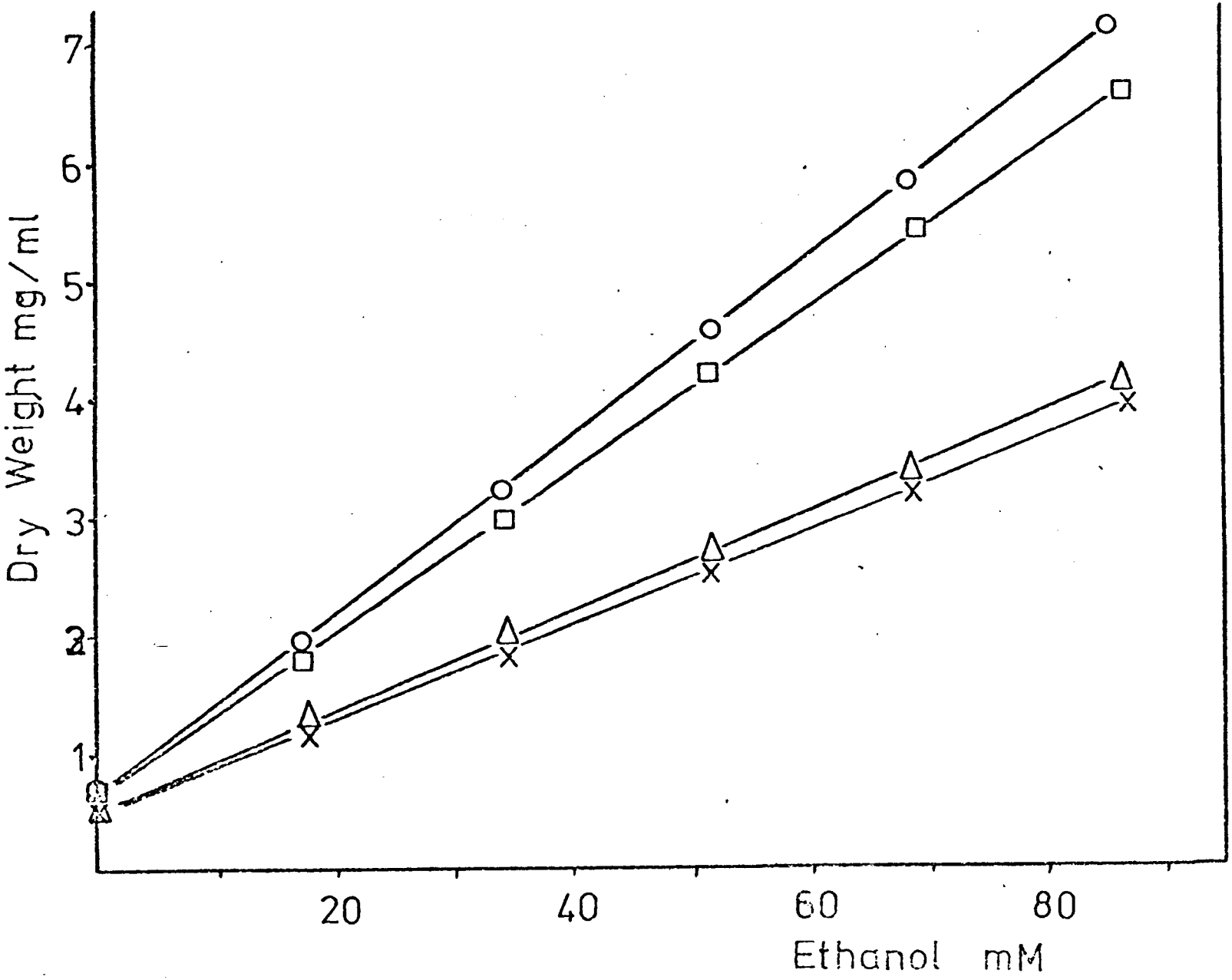
Growth of S. cerevisiae on Glucose.

As is well known, S. cerevisiae is repressed by glucose (Jayaraman et al, 1966). In aerobic culture on high concentrations of glucose Saccharomyces sp. grow firstly by fermentation and then there is a definite stationary phase while the organisms undergo respiratory adaptation in order to utilise the ethanol produced. It is evident (Figure 2.8) that, compared to the wild type, all the $TTFB^R$ mutants were repressed by a low concentration of glucose (0.4% w/v); most having an inflection in the growth curve at a cell concentration of about 1.0 mg dry weight per ml. The subsequent growth phase on ethanol for all mutants examined was comparable with the previous results (Figures 2.1 - 2.3) in that the growth rates were always less than that of the wild type. The mutant strains were unaffected in the fermentative phase of growth except for D22-DCS11 which was defective. The "1799"^{R} mutants, D22-CB9 and D22-CB19, were also slightly repressed (Figure 2.9) and similarly the TET^R mutant D22-EC1. However, the mutant D22-EC2 had the same growth curve as the wild type, showing no evidence of repression. Again all these strains were relatively unaffected in the initial fermentative stage of growth. Accurate generation times for growth on glucose were not obtained in these experiments. However in the latter stages of growth the rates approached those of the cells grown on ethanol. In a few cases experiments were also done employing higher concentrations of glucose (2.0% w/v). Under these conditions glucose repression was always seen and for the mutants the time taken for respiratory adaptation was longer than that required by the wild type.

Molar Growth Yields of S. cerevisiae on Ethanol or on Glucose.

As indicated in previous Figures few of the mutants had the same growth yields as the wild type on either ethanol or glucose. A more detailed examination of the molar growth yields of selected strains under more rigorously defined conditions was therefore undertaken. As shown in Figure 2.10 the growth yields of various strains on ethanol mostly follow the general trends indicated in Figures 2.1 - 2.3. However D22-CB19 proved to be an exception, having a lower growth yield than expected. A similar result is seen for the TET^R strains, D22-EC1 and D22-EC2, (Figure 2.11) neither strain having the same characteristics as the wild type (in contrast to Figure 2.3).

Figure 2.10



Growth yields of S. cerevisiae in batch culture on ethanol.

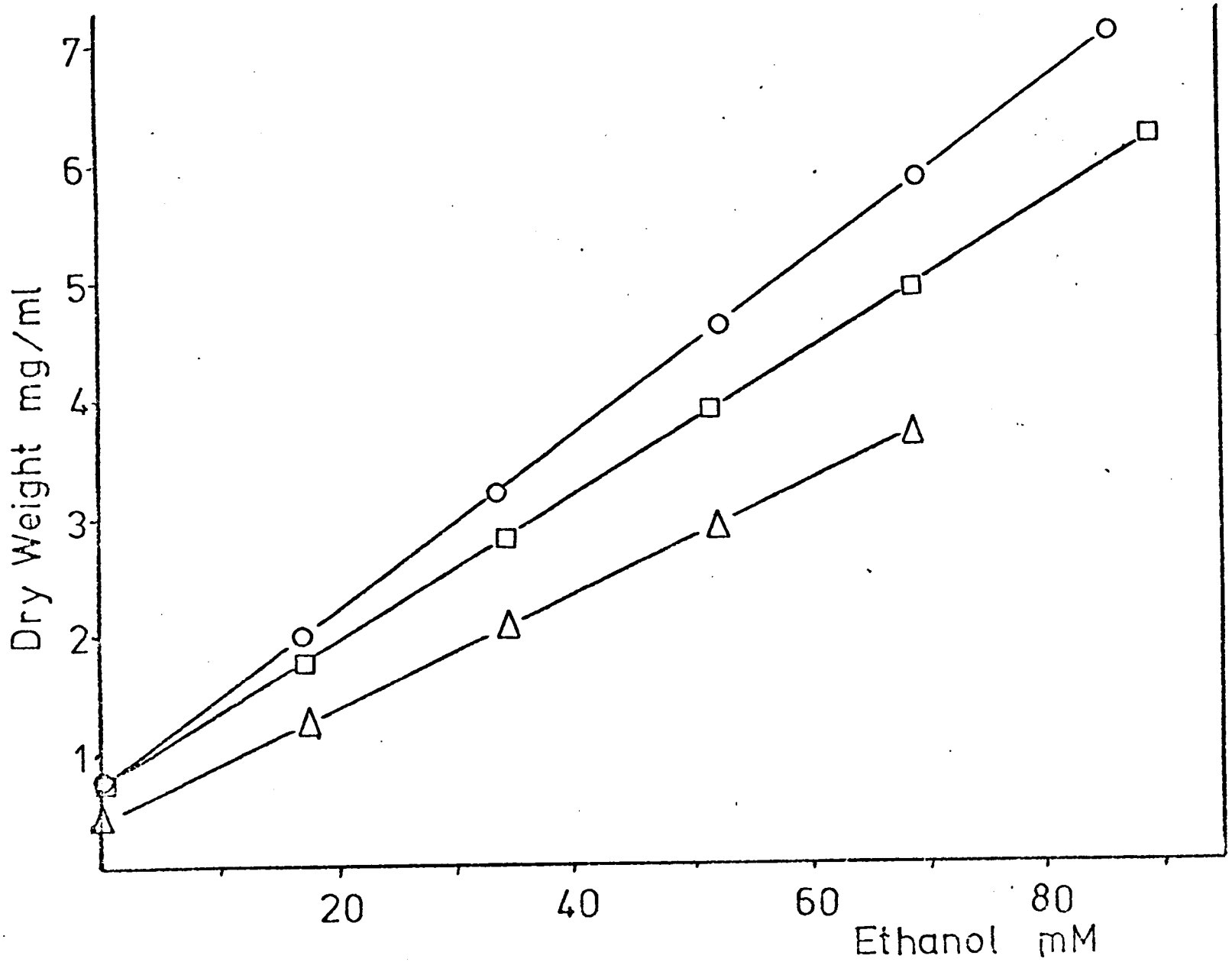
O - O D22; D22 - CB9; D22 - DCS

□ - □ D22 - CB9.

Δ - Δ D22 - DCS9.

X - X D22 - DC5; D22 - DC9.

Figure 2.11



Growth yields of S. cerevisiae in batch culture on ethanol.

O - O D22.

□ - □ D22 - EC2.

Δ - Δ D22 - EC1.

TABLE 2.7

Growth yields of S. cerevisiae on ethanol.

Strain	Type	Class	$Y_{Ethanol}$	Y_{ATP}	Phosphorylation Efficiency	
					Theory	Experiment
D22	wild type	-	75.5	6.3	12	7.2
D22-DCS12	$TTFB^R$	2	75.5	6.3	12	7.2
D22-CB9	"1799"^{R}	1	75.5	6.3	12	7.2
D22-CB19	"1799"^{R}	2	70.8	5.9	12	6.8
D22-EC2	TET^R	3	60.0	5.0	12	5.7
D22-DCS9	$TTFB^R$	3	51.0	4.3	12	4.9
D22-EC1	TET^R	2	48.4	4.1	12	4.6
D22-DC9	$TTFB^R$	2	39.5	3.3	12	3.8
D22-DC5	$TTFB^R$	3	39.5	3.3	12	3.8

Notes

$Y_{substrate}$ = molar yield coefficient = $\frac{\text{gm dry weight cells}}{\text{mole substrate}}$

Y_{ATP} = molar yield coefficient = $\frac{\text{gm dry weight cells}}{\text{mole ATP}}$

Theoretical phosphorylation efficiency = $\frac{\text{moles ATP}}{\text{moles substrate}}$

Experimental phosphorylation efficiency = $\frac{Y_{substrate}}{\text{Elsden constant}}$

Elsden constant = 10.5 gm dry weight/mole ATP

Two broad divisions are seen in Table 2.7. There are strains having growth yields comparable to the wild type ie. D22-DCS12, D22-CB9, D22-CB19 and D22-EC2 and those having rather less ie. D22-DC5, D22-DC9, D22-DCS9 and D22-EC1. These results correlate well with Table 2.6, but growth yields of D22-DCS11 were not examined.

Corresponding data with glucose as oxidisable substrate are shown in Figures 2.12 and 2.13. In contrast to the results on ethanol, no strain had the same growth yield as the wild type, which is in line with the growth curves shown in Figures 2.8 and 2.9. However, D22-EC2 did not have the same growth yield as the wild type. Again there are the same broad divisions (Table 2.8) into those strains having yields comparable with that of the wild type ie. D22-DCS12, D22-CB9, D22-CB19 and D22-EC2 and those having much less ie. D22-DC5, D22-DC9, D22-DCS9 and D22-EC1.

In no case was the growth yield for a mutant strain higher than that of the wild type. In Figures 2.10 - 2.13 it can be seen that only a small amount of growth occurs on casein hydrolysate plus yeast extract, in the absence of any added oxidisable substrate. It is therefore assumed that only glucose or ethanol are used as carbon scource to provide energy and that little or none of the casein hydrolysate is used for this purpose. Figures 2.10 -2.13 indicate the relationship between cell yield and the concentration of substrate used as energy scource. The slope of the graph gives the molar yield coefficient ($Y_{substrate}$); and the ATP coefficient (Y_{ATP}) is calculated assuming the following stoichiometries for ATP formation:-

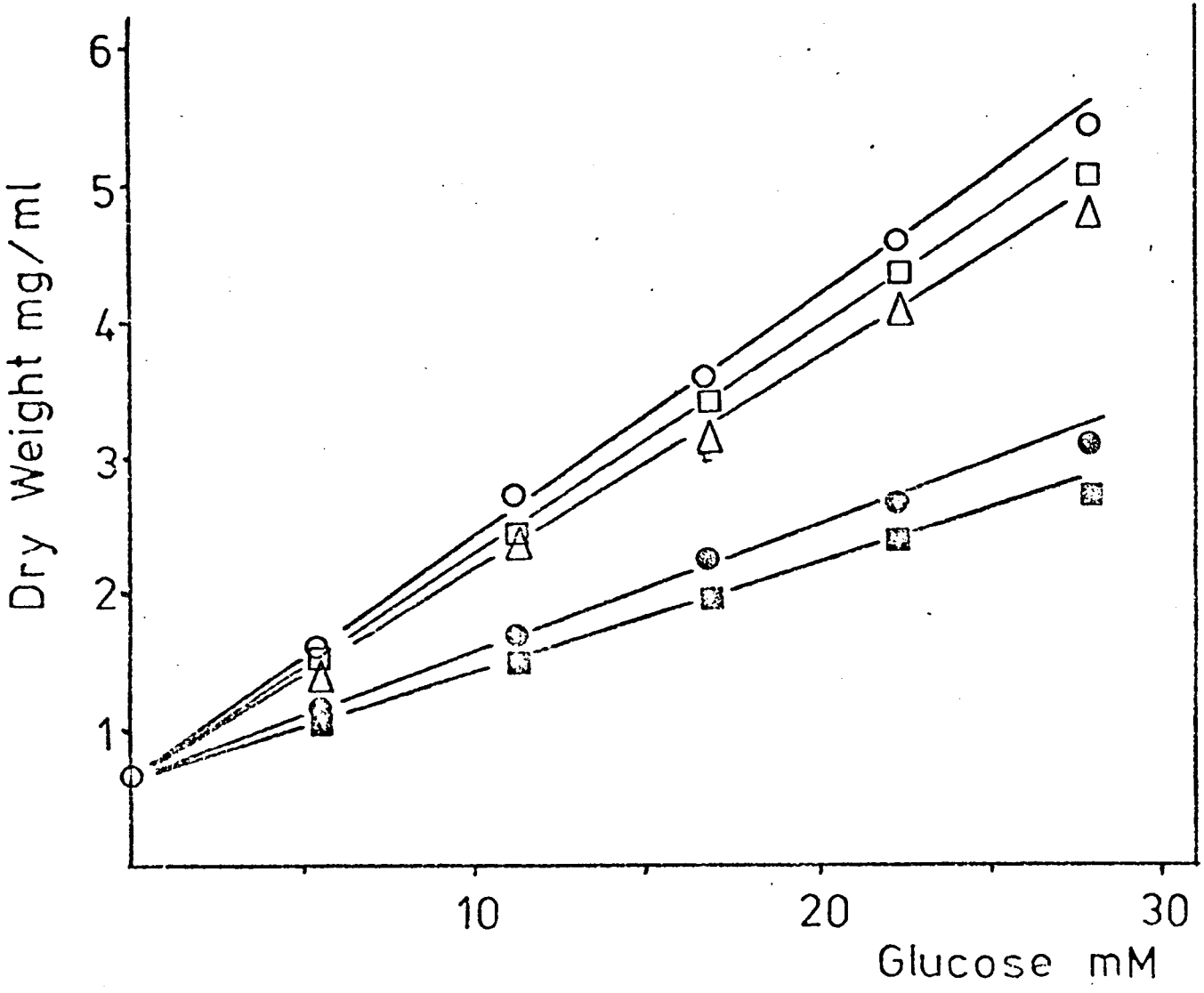
Ethanol $C_2H_5OH + 3O_2 + 12ADP + 12Pi = 3H_2O + 2CO_2 + 12ATP$

Glucose $C_6H_{12}O_6 + 6O_2 + 28ADP + 28Pi = 6H_2O + 6CO_2 + 28ATP$

In formulating these equations it has been assumed that S. cerevisiae mitochondria lack Site I phosphorylation (see Chapter 3).

The average ATP coefficient (Y_{ATP} = 10.5 gm dry weight per mole ATP) has been calculated for several microorganisms (Bauchop and Elsden, 1960) and since then has been assumed to be relatively constant (Forest and Walker, 1971). This assumption is now open to question (Stouthamer and Bettenhausen, 1973). However for the purposes of comparison in Tables 2.7 and 2.8 the proposals of Stouthamer and Bettenhausen are ignored and are discussed later.

Figure 2.12



Growth yields of S. cerevisiae in batch culture on glucose.

O - O D22.

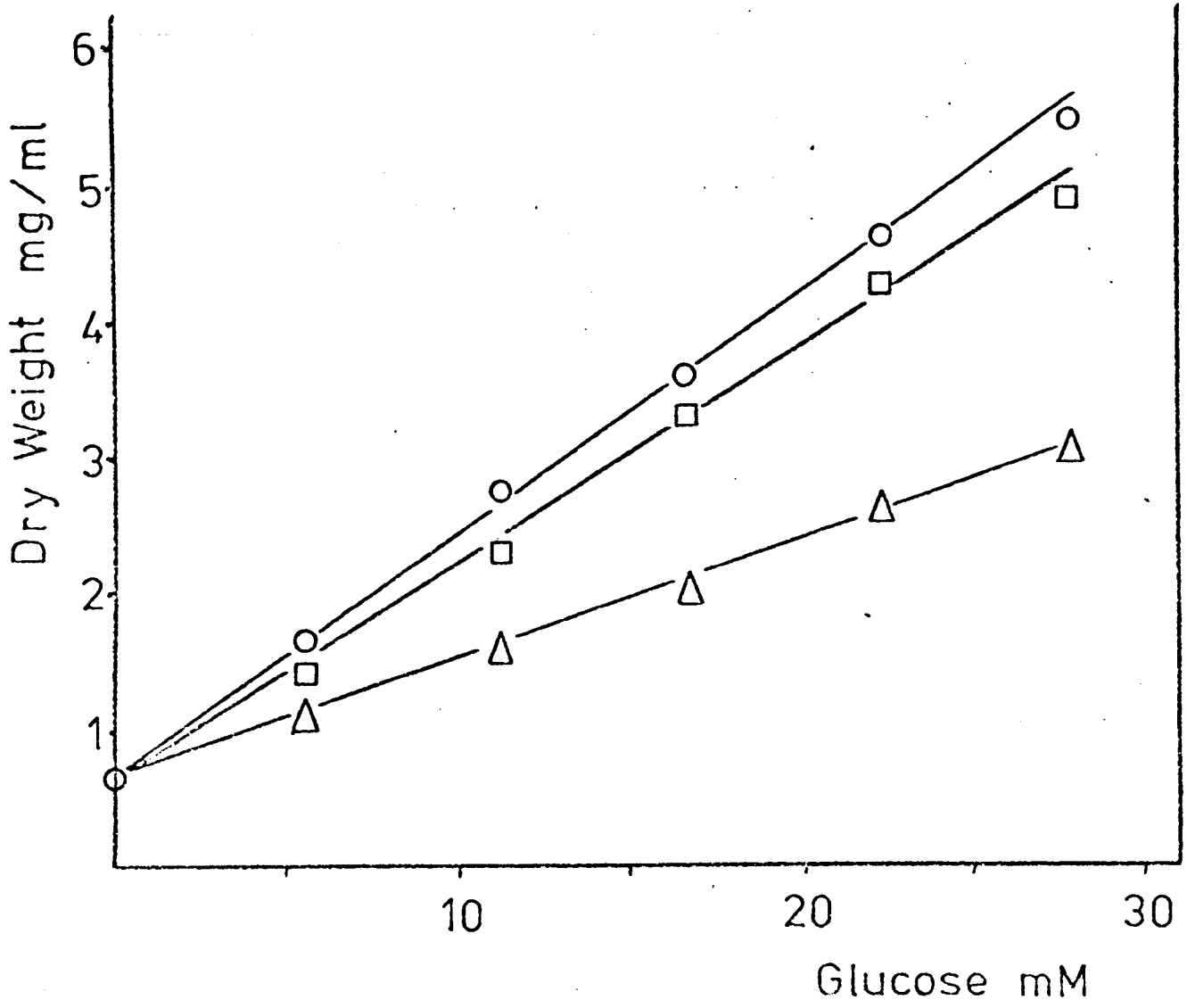
□ - □ D22 - CB9.

Δ - Δ D22 - CB19; D22 - DCS12.

● - ● D22- DCS9.

■ - ■ D22 - DC5; D22 - DC9.

Figure 2.13



Growth of S. cerevisiae in batch culture on glucose.

O - O D22.

□ - □ D22 - EC2.

Δ - Δ D22 - EC1.

TABLE 2.8

Growth yields of S. cerevisiae on glucose.

Strain	Type	Class	$Y_{Glucose}$	Y_{ATP}	Phosphorylation Efficiency	
					Theory	Experiment
D22	wild type	-	176.0	6.3	28	16.8
D22-EC2	TET^R	3	154.0	5.5	28	14.7
D22-CB9	"1799"^{R}	1	154.0	5.5	28	14.7
D22-CB19	"1799"^{R}	2	126.5	4.5	28	12.0
D22-DCS12	$TTFB^R$	2	126.5	4.5	28	12.0
D22-DCS9	$TTFB^R$	3	91.0	3.3	28	8.7
D22-EC1	TET^R	2	91.0	3.3	28	8.7
D22-DC9	$TTFB^R$	2	79.4	2.8	28	7.6
D22-DC5	$TTFB^R$	3	79.4	2.8	28	7.6

Notes

$Y_{substrate}$ = molar yield coefficient = $\frac{\text{gm dry weight cells}}{\text{mole substrate}}$

Y_{ATP} = molar yield coefficient = $\frac{\text{gm dry weight cells}}{\text{mole ATP}}$

Theoretical phosphorylation efficiency = $\frac{\text{moles ATP}}{\text{moles substrate}}$

Experimental phosphorylation efficiency = $\frac{Y_{substrate}}{\text{Elsden constant}}$

Elsden constant = 10.5 gm dry weight/mole ATP

The ratio of the molar yield coefficient on a given substrate to the average ATP coefficient is denoted as the "phosphorylation coefficient" and represents moles ATP formed from one mole of substrate under the conditions given. The values of $Y_{substrate}$ for any strain have not been corrected in these results for the amount of substrate assimilated by the cells. It is assumed that this is the same in all cases.

From Table 2.7, the mutants fall into two groups; those with $Y_{ethanol}$ equal to 60-75 gm dry weight per mole and those with $Y_{ethanol}$ equal to 39.5-51.0 gm dry weight per mole. This trend is repeated in Table 2.8 with one group having $Y_{glucose}$ at 126.5 - 176.0 gm dry weight per mole and the other at 79.4 - 91 gm dry weight per mole. In no case does the Y_{ATP} value approach the theoretical value on either glucose or ethanol and similarly for the "phosphorylation efficiencies". No mutant strain has a higher Y_{ATP} value than the wild type.

Relative Cytochrome Contents of S. cerevisiae Cells From Difference Spectroscopy at 77°K.

The cytochrome profile of cells of S. cerevisiae, strain D22, wild type and selected drug or uncoupler resistant mutants have been examined by difference spectroscopy. Measurements have been made at low temperature (77°K utilising liquid nitrogen), and also at room temperature.

The low temperatures permit increased resolution of the different components while the room temperature spectra are necessary in order to obtain quantitative data.

The use of low temperature spectroscopy in the analysis of mitochondrial cytochromes has been described by Chance, (1957). Certainly the contribution of cytochrome c_1 to the absorption spectrum is most easily seen by this technique. Antimycin inhibits mitochondrial electron transport between cytochromes b and c_1 (Slater, 1973). This inbibitor has therefore been used to provide further resolution of the absorption spectrum of the cytochromes present in the yeast cells.

The low temperature, reduced minus oxidised cytochrome spectrum of S. cerevisiae, strain D22 fermenter grown cells, grown on ethanol (0.5% v/v), to early stationary phase, is shown in Figure 2.14. This spectrum is typical of aerobically grown non-repressed Saccharomyces yeast, with a relatively large amount of cytochrome c (550 nm) and a small amount of cytochrome a a_3

(605 nm). These characteristics were even more noticeable in the Soret region where the cytochrome a a_3 absorption peak (445 nm) appeared as a shoulder on the larger peak due to cytochrome c (415 nm) and the b-type cytochromes (430 nm). The absorption due to cytochrome c_1 appeared as a shoulder at 554 nm on the cytochrome c absorption. Estabrook, (1956) has reported that the α - band of the cytochrome c absorption spectrum at 77° K is shifted towards shorter wavelengths and split into two peaks ie. $C_{\alpha 1}$ at 549 nm and $C_{\alpha 2}$ at 546 nm. In Figure 2.14 neither of these effects was observed. Under the conditions used to record this spectrum a shift of $\pm$ 1 nm would not be measureable and the cytochrome $C_{\alpha 2}$ peak (which appears as a small shoulder) was probably missed owing to the use of a relatively high scan speed. Although there are several b-type cytochromes present in yeast cells (Sato et al, 1972) only one absorption peak (558 nm) is seen in Figure 2.14.

Under the correct conditions the use of antimycin can bring about reduction of all the b-type cytochromes in the yeast cell. In Figure 2.15 this inhibitor has been used in and attempt to obtain an absorption spectrum at 77° K due to the b-type cytochromes alone. Under the assay conditions used here it was not possible to maximally reduce all the b-type cytochromes (Sato et al, 1972). There was only one Soret peak (425 nm) with very little detail in the region of the β absorption bands. A large absorption peak at 558 nm was present with shoulders at 553 nm and at 548 nm. It is also possible for antimycin to enable the recording of the absorption spectrum due to cytochromes c_1, c and a a_3 with little or no interference from b-type cytochromes (Figure 2.16). As a result of the block on electron transport the measurable amounts of these cytochromes were increased.

S. cerevisiae, strain D22, cells were also cultured aerobically on ethanol (0.5 % v/v) in 2 ltr. conical flasks. The reduced minus oxidised absorption spectrum at 77° K of early stationary phase cells is shown in Figure 2.17. Comparison with Figure 2.14 shows considerable differences in detail. There were increases in the amounts of all cytochromes relative to cytochrome c and this was especially so for cytochrome a a_3. It is therefore concluded that the oxygenation of the medium in the flask culture was much better than in the fermenter.

It is not possible to obtain quantitative data from measurements of cytochrome difference spectra at 77° K because in every case the extinction coefficients are unknown. However the cytochrome contents of the cells can be compared on a relative basis. The difference spectra at 77° K for S. cerevisiae, strain D22

FIGURE 2.14

Reduced minus oxidised cytochromedifference spectrum of S. cerevisiae, Strain D22, at 77°K. The cells were fermenter grown, to stationary phase, on 0.5% (v/v) ethanol. Cell concentration was 27 mg dry weight/ml.

FIGURE 2.15

Cytochrome difference spectrum of S. cerevisiae, strain D22, at 77°K. The cells were fermenter grown to stationary phase on 0.5% (v/v) ethanol. For the sample cuvette, 30 μg antimycin A, 10 μg ethanol and 5 μl hydrogen peroxide (100 vol) were added to 1 ml cell suspension (27 mg dry weight/ml). An aliquot was put in the cuvette and frozen. For the reference 3.9 μM TTFB and 5 μl hydrogen peroxide (100 vol) were added to 1 ml cell suspension and an aliquot again taken for examination.

FIGURE 2.16

Cytochrome difference spectrum of S. cerevisiae strain D22, at 77°K. The cells were fermenter grown to stationary phase on 0.5% (v/v) ethanol. For the sample cuvette, a few grains of sodium dithionite together with 3.9 μM TTFB were added to 1 ml cell suspension (22 mg dry weight/ml). An aliquot was then put in the cuvette and frozen. For the reference, 30 μg Antimycin A, 10 μl ethanol and 5 μl hydrogen peroxide (100 vol) were added to 1 ml cell suspension and an aliquot again taken for examination.

FIGURE 2.17

Reduced minus oxidised cytochromedifference spectrum of S. cerevisiae, strain D22, at 77°K. The cells were grown to stationary phase on 0.5 % (v/v) ethanol in shake flask culture. The cell concentration was 11 mg dry weight/ml.

Figure 2.14

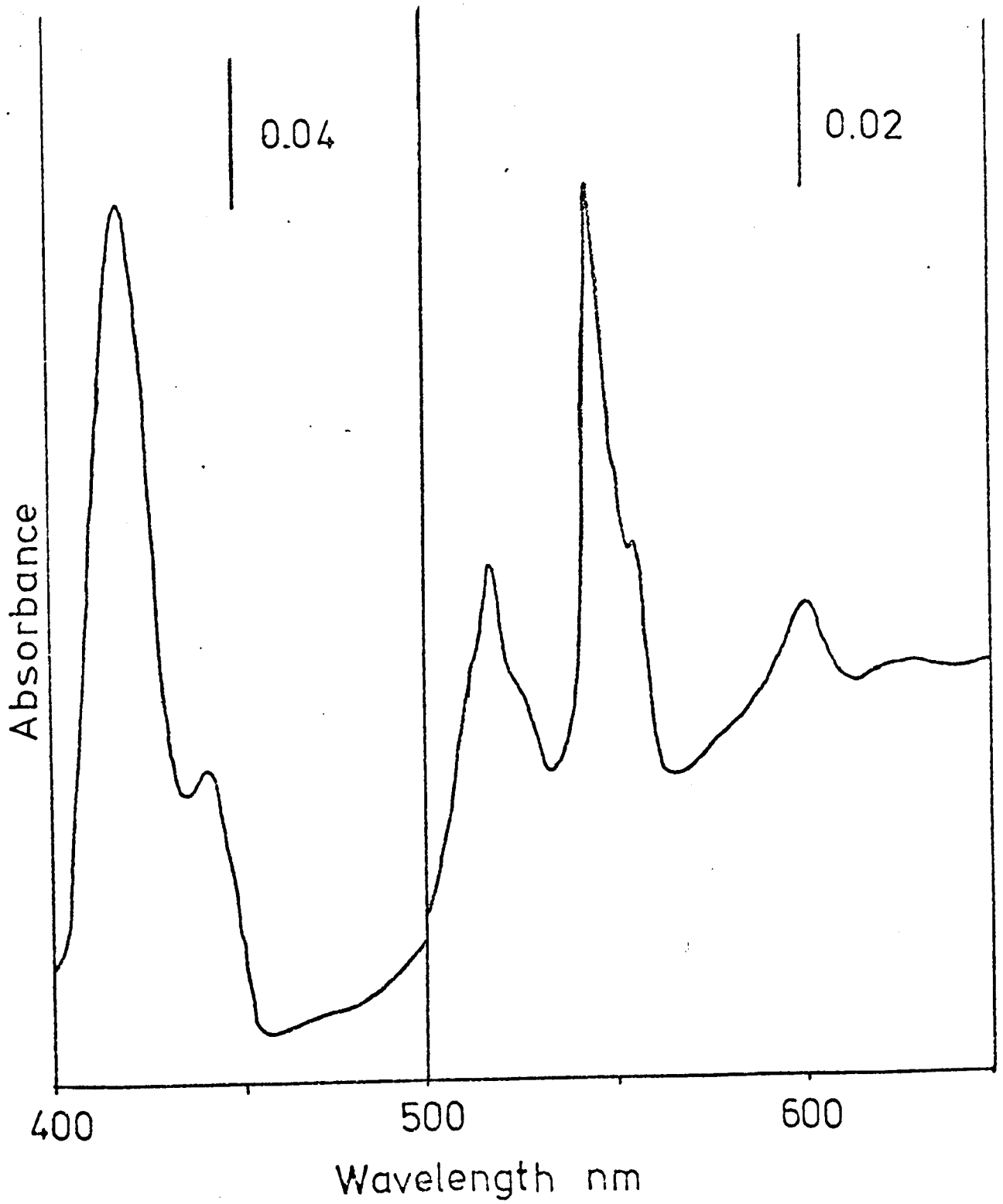


Figure 2.15

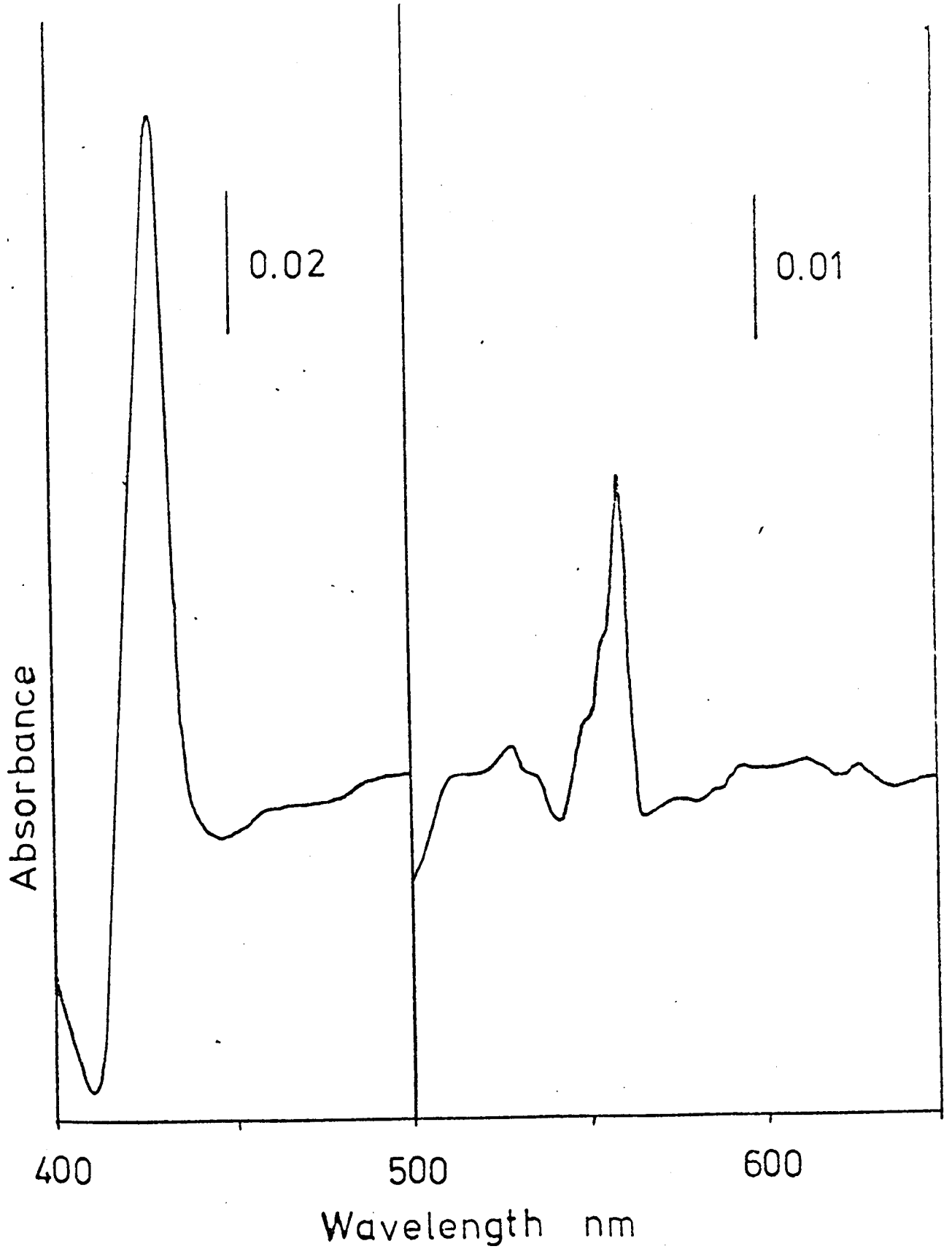


Figure 2.16

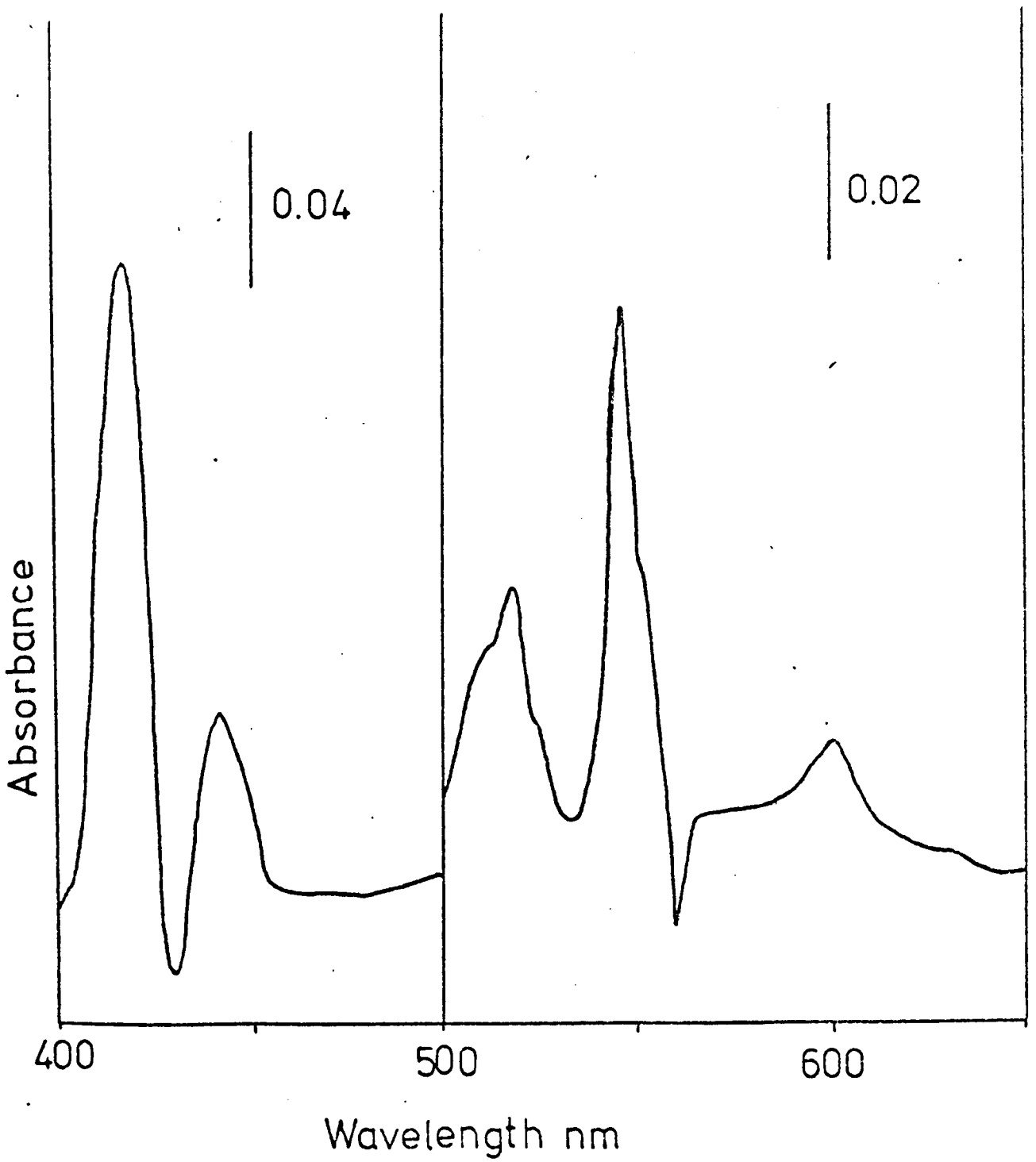


Figure 2.17

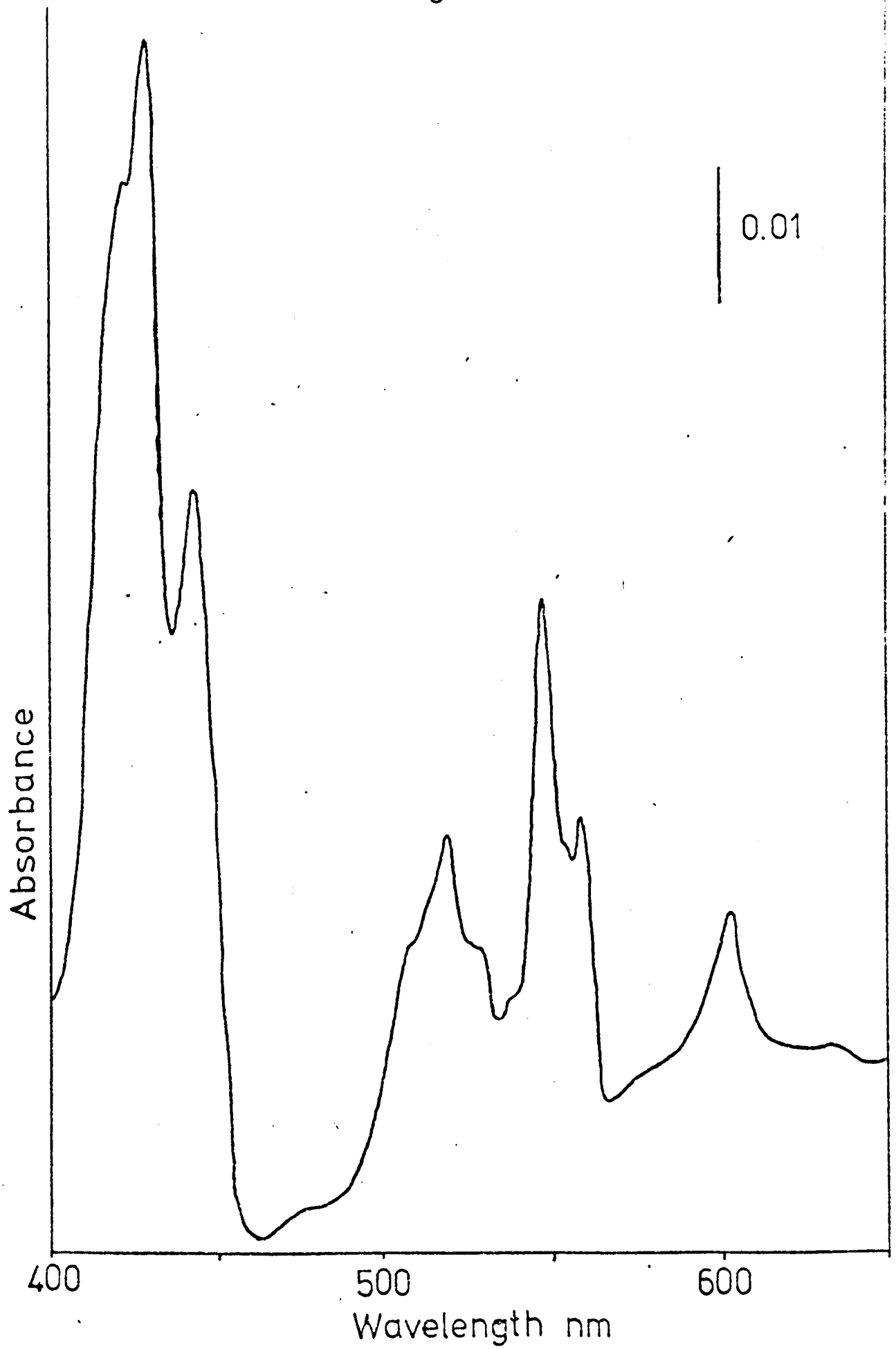


TABLE 2.9

Relative cytochrome contents (at 77°K) of S. cerevisiae cells grown on ethanol.

	Strain	Growth Conditions	Additions to sample cuvette	Additions to reference cuvette	Concentration ratio				Relative percentage (to cytochrome c).			
					c	c_1	b	aa_3	$\frac{c}{c}$	$\frac{c_1}{c}$	$\frac{b}{c}$	$\frac{aa_3}{c}$
1.	D22	Fermenter	Dithionite	TTFB+H_2O_2	3.7	1.9	1.0	0.3	100	50.4	26.8	9.0
2.	D22	Fermenter	Ethanol+H_2O_2 + Antimycin	TTFB+H_2O_2	-	-	1.1	-				
3.	D22	Fermenter	Dithionite	Ethanol + H_2O_2 + Antimycin	3.1	1.4	-	0.42	100	44.2	-	13.5
4.	D22	Shake flask	Dithionite	TTFB+H_2O_2	1.5	0.6	0.6	0.4	100	38.2	36.4	27.8
5.	D22-DCS12	Fermenter	As 1.	As 1.	3.7	1.8	1.1	0.3	100	48.1	29.6	6.8
6.	D22-DCS12	Fermenter	As 2.	As 2.	-	-	1.9	-				
7.	D22-DCS12	Fermenter	As 3.	As 3.	3.9	1.5	-	0.5	100	38.0	-	12.8
8.	D22-CB19	Fermenter	As 1.	As 1.	2.7	1.6	0.8	0.3	100	59.0	31.0	9.4
9.	D22-CB19	Fermenter	As 2.	As 2.	-	-	1.0	-				
10.	D22-CB19	Fermenter	As 3.	As 3.	2.5	0.7	-	0.3	100	30.4	-	13.6

Notes

1. Only cytochrome $b_{560\ nm}$ is quoted in this Table.
2. It is assumed that all extinction coefficients are equally enhanced and that they stay in the same ratio as those at room temperature ie. ϵ_{mM} = 19 : 17.5 : 22 : 24 = cytochrome c : c_1 : b : aa_3 (see Methods).
3. Nos. 1-4 in this Table are analyses of Figures 2.14-2.17 respectively.
4. Cell concentrations for D22-DCS12 spectra were 24 mg dry weight/ml and for D22-CB19, 18 mg dry weight/ml.
5. These results were obtained at the Johnson Foundation.

(Figures 2.14 - 2.17) and the uncoupler resistant mutants D22-DCS12 and D22-CB19 (not shown) have been analysed in this way (Table 2.9). There is no evidence for the appearance of any new species of cytochrome in the mutants. In going from fermenter grown to flask grown cells in the case of the wild type strain, there was an increase in the relative content of cytochrome $a a_3$. Antimycin produced some increase in the measurable amounts of the cytochromes in all cases. Although the content of cytochrome $a a_3$ in fermenter grown cells was less than maximal, probably owing to a lower oxygen tension, the mutant strain D22-DCS12 has relatively less cytochrome $a a_3$ than the wild type cells grown under these same conditions. Table 2.9 provides no measurement of the actual amounts of each cytochrome present in the yeast cell. An increase in, for example, the $\frac{aa3}{c}$ ratio could be either due to an increase in the amount of cytochrome $a a_3$ or to a decrease in the amount of cytochrome c.

Cytochrome Contents of S. cerevisiae Cells from Difference Spectroscopy.

The measurements shown in Figures 2.14 to 2.16 were repeated at room temperature for strain D22 (Figures 2.18 - 2.20 respectively), and also for the uncoupler resistant mutants D22-DCS12 and D22-CB19 (not shown). All cells were again fermenter grown to early stationary phase on 0.5% (v/v) ethanol. The measurable amounts of cytochromes on a dry weight basis were of the same order in all cases (Table 2.10). Using antimycin, the contribution of the b-type cytochromes to the absorption spectrum was increased, especially for D22-DCS12. The measurement of cytochrome c was relatively unaffected by this technique but the absorption peak due to cytochrome $a a_3$ was increased in all cases. Although growth conditions in the fermenter are less than optimal for cytochrome synthesis, these data indicate that both the mutants have consistently lower levels of cytochrome $a a_3$ than the wild type cells grown under the same conditions. The use of antimycin reduces the possibility of error due to incomplete oxidation of the cytochrome $a a_3$ in the reference sample. When this inhibitor was used there was still a greater cytochrome $a a_3$ absorption peak in the wild type compared with that of either of the mutants.

A better comparison of the cytochrome content of the S. cerevisiae cells grown aerobically on ethanol can be made if all the strains are grown in shake flask culture. The increased aeration of the medium should promote maximal synthesis of cytochromes, especially cytochrome $a a_3$ (Figure 2.17). The reduced minus oxidised spectrum of S. cerevisiae, strain D22 grown under

FIGURE 2.18

Reduced minus oxidised cytochrome difference spectrum of S. cerevisiae, strain D22. The cells were grown in a fermenter to stationary phase on 0.5% (v/v) ethanol. The cell concentration was 29 mg dry weight/ml.

FIGURE 2.19

Cytochrome difference spectrum of S. cerevisiae, strain D22. The cells were grown in a fermenter to stationary phase on 0.5% (v/v) ethanol. The sample cuvette (1cmlight path) consisted of 2.5 ml cell suspension (22 mg dry weight/ml) in 60% (w/v) sorbitol, 50 mM potassium phosphate buffer, pH 7.0 with 25 µl ethanol, 75µg antimycin A and 10 µl hydrogen peroxide (100 vol). Additions to the reference cuvette were 3.9 µM TTFB and 10 µl hydrogen peroxide (100 vol).

FIGURE 2.20

Cytochrome difference spectrum of S. cerevisiae, strain D22. The cells were grown in a fermenter to stationary phase on 0.5% (v/v) ethanol. The sample cuvette (1 cm light path) consisted of 2.5 ml cell suspension (22 mg dry weight/ml) in 60% (w/v) sorbitol, 50 mM potassium phosphate buffer pH 7.0 with 2.9 µM TTFB. The cytochromes were reduced by addition of a few grains of sodium dithionite. The reference cuvette contained 2.5 ml cell suspension with 25 µl ethanol, 75 µg antimycin A and 10 µl hydrogen peroxide (100 vol).

Figure 2.18

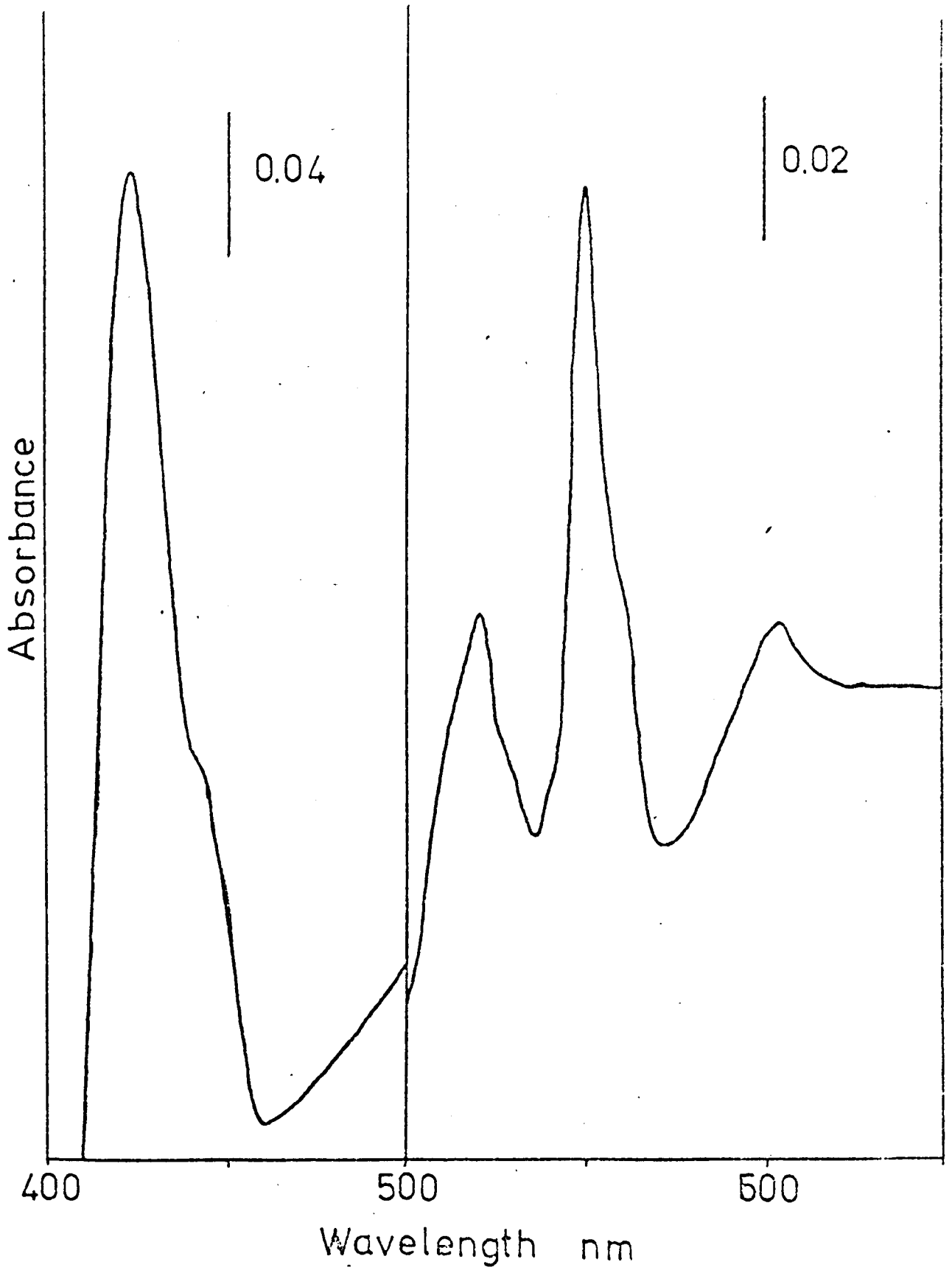


Figure 2.19

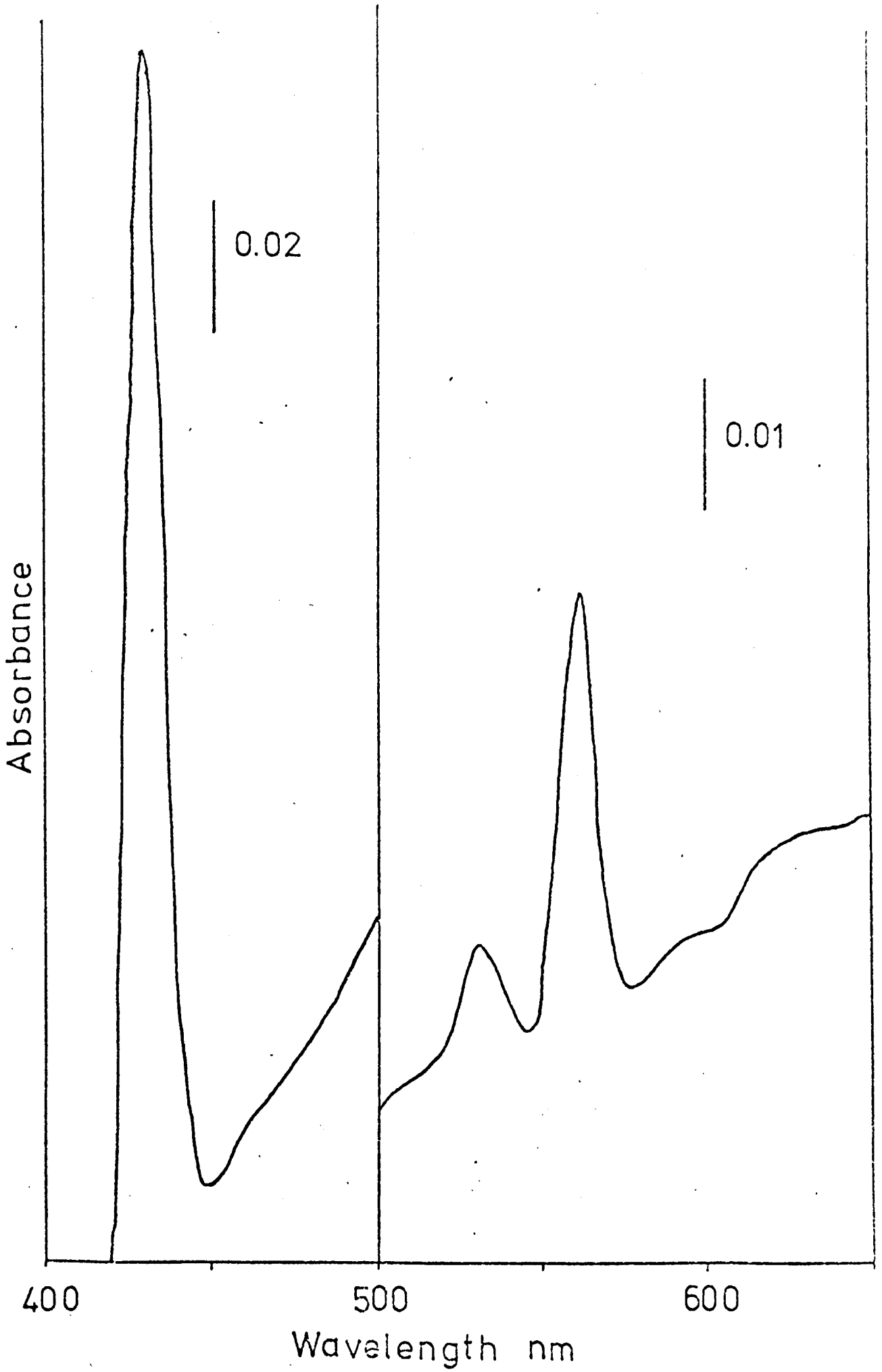


Figure 2.20

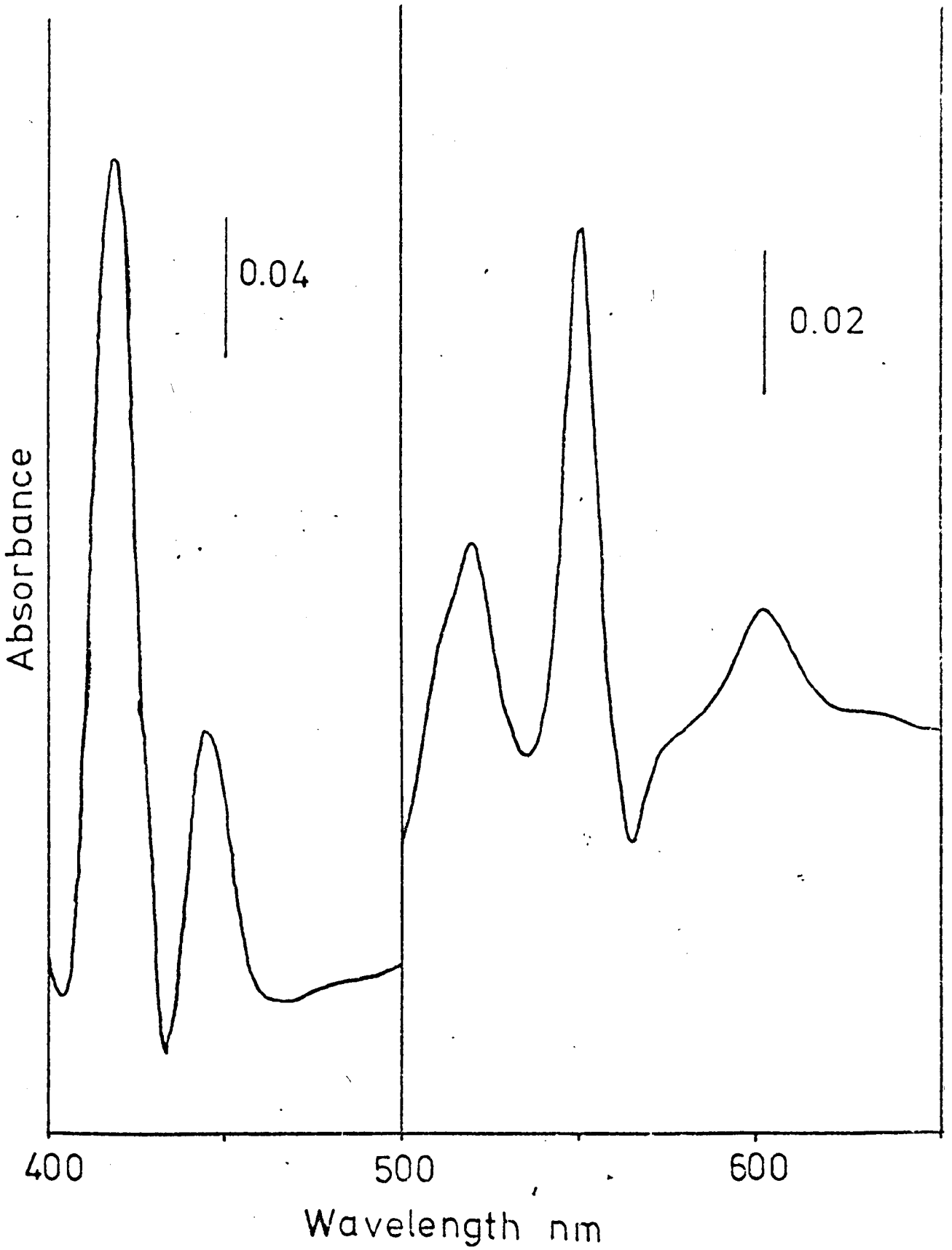


TABLE 2.10

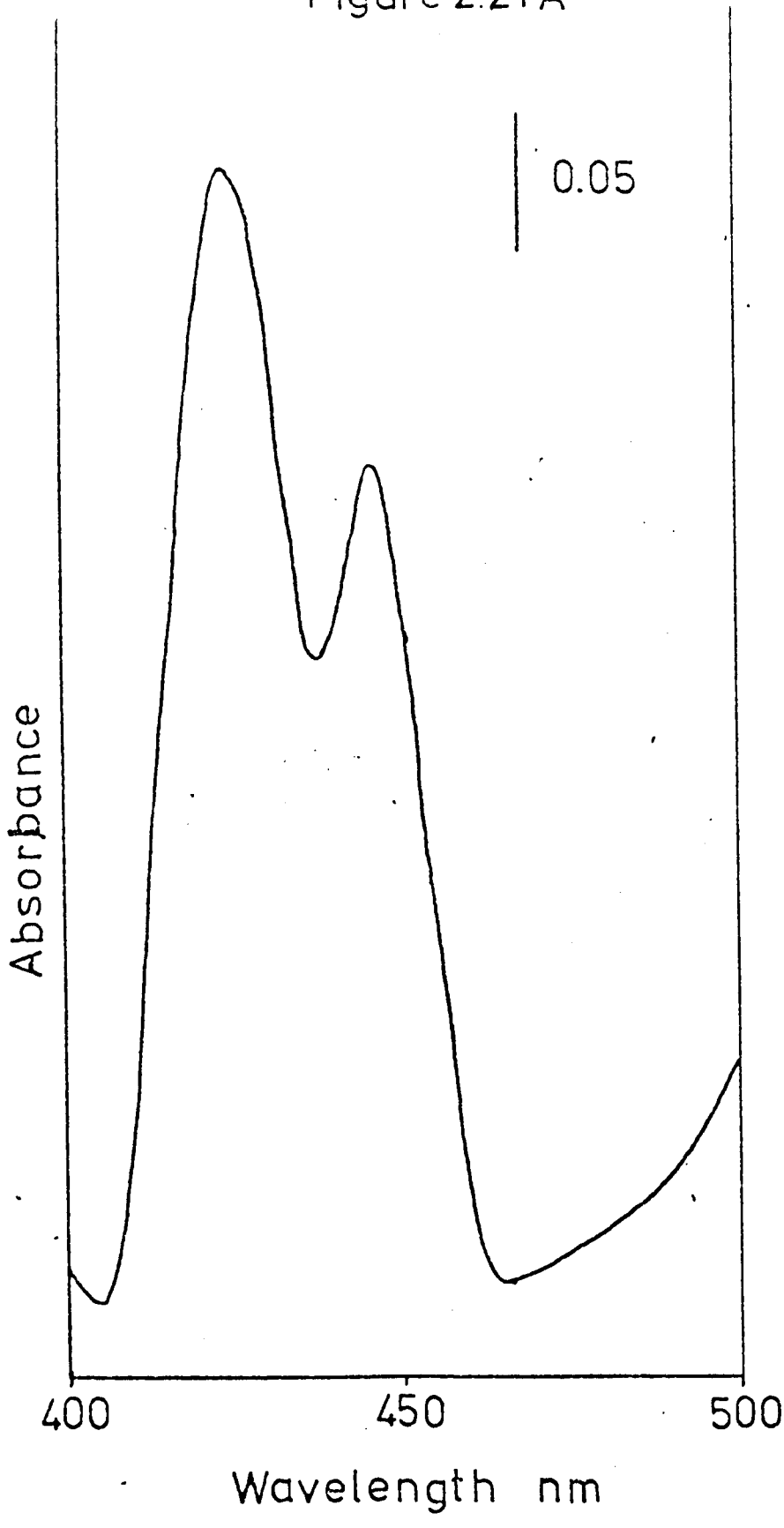
Cytochrome contents of S. cerevisiae cells grown on ethanol.

	Strain	Growth Conditions	Additions to sample cuvette	Additions to reference cuvette	nmole cytochrome / mg dry weight			Relative percentage (to cytochrome c)		
					c	b	aa_3	$\frac{c}{c}$	$\frac{b}{c}$	$\frac{aa_3}{c}$
1.	D22	Fermenter	Dithionite	TTFB + H_2O_2	0.15	0.046	0.013	100	30.0	8.5
2.	D22	Fermenter	Ethanol + H_2O_2 + Antimycin	TTFB + H_2O_2	-	0.06	-			
3.	D22	Fermenter	Dithionite	Ethanol + H_2O_2 + Antimycin	0.16	-	0.027	100	-	16.5
4.	D22-DCS12	Fermenter	As 1.	As 1.	0.16	0.04	0.012	100	25.9	7.9
5.	D22-DCS12	Fermenter	As 2.	As 2.	-	0.114	-			
6.	D22-DCS12	Fermenter	As 3.	As 3.	0.16	-	0.019	100	-	11.9
7.	D22-CB19	Fermenter	As 1.	As 1.	0.15	0.047	0.011	100	30.6	7.0
8.	D22-CB19	Fermenter	As 2.	As 2.	-	0.046	-			
9.	D22-CB19	Fermenter	As 3.	As 3.	0.15	-	0.018	100	-	12.1

Notes

1. Nos. 1 - 3 in this Table are analyses of Figures 2.18 - 2.20 respectively.
2. Cell concentrations for D22-DCS12 spectra were 27 mg dry weight/ml; and for D22-CB19, 18 mg dry weight/ml.
3. 1 cm light path cells were used. Extinction coefficients and wavelength pairs are listed in Methods.
4. These results were obtained at the Johnson Foundation.

Figure 2.21 A



Reduced minus oxidised cytochrome difference spectrum of S. cerevisiae, strain D22. The cells were grown to stationary phase on 0.5% (v/v) ethanol in shake flask culture. Cell concentration was 42 mg dry weight/ml.

Figure 2.21 B

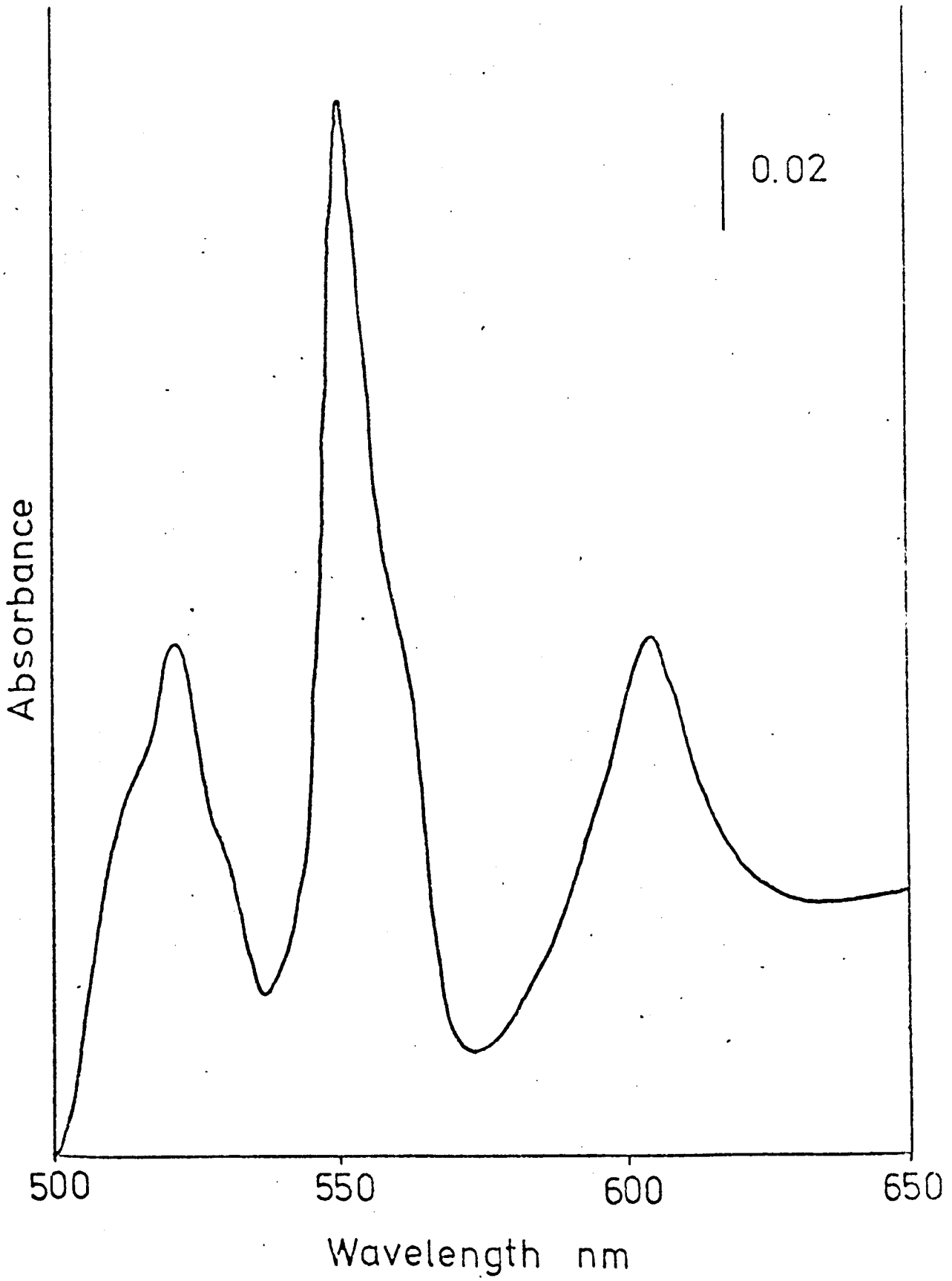


FIGURE 2.22

Reduced minus oxidised cytochrome difference spectra of S. cerevisiae.
Cells were grown to stationary phase on 0.5% (v/v) ethanol in shake flask culture.
D22-DCS9, 29 mg dry weight/ml; D22-DCS12, 39.5 mg dry weight/ml.

FIGURE 2.23

Reduced minus oxidised cytochrome difference spectra of S. cerevisiae.
Cells were grown to stationary phase on 0.5 % (v/v) ethanol in shake flask culture.
D22-DC5, 34 mg dry weight/ml; D22-DC9, 38 mg dry weight/ml; D22-DCS11,
20 mg dry weight/ml.

Figure 2.22A

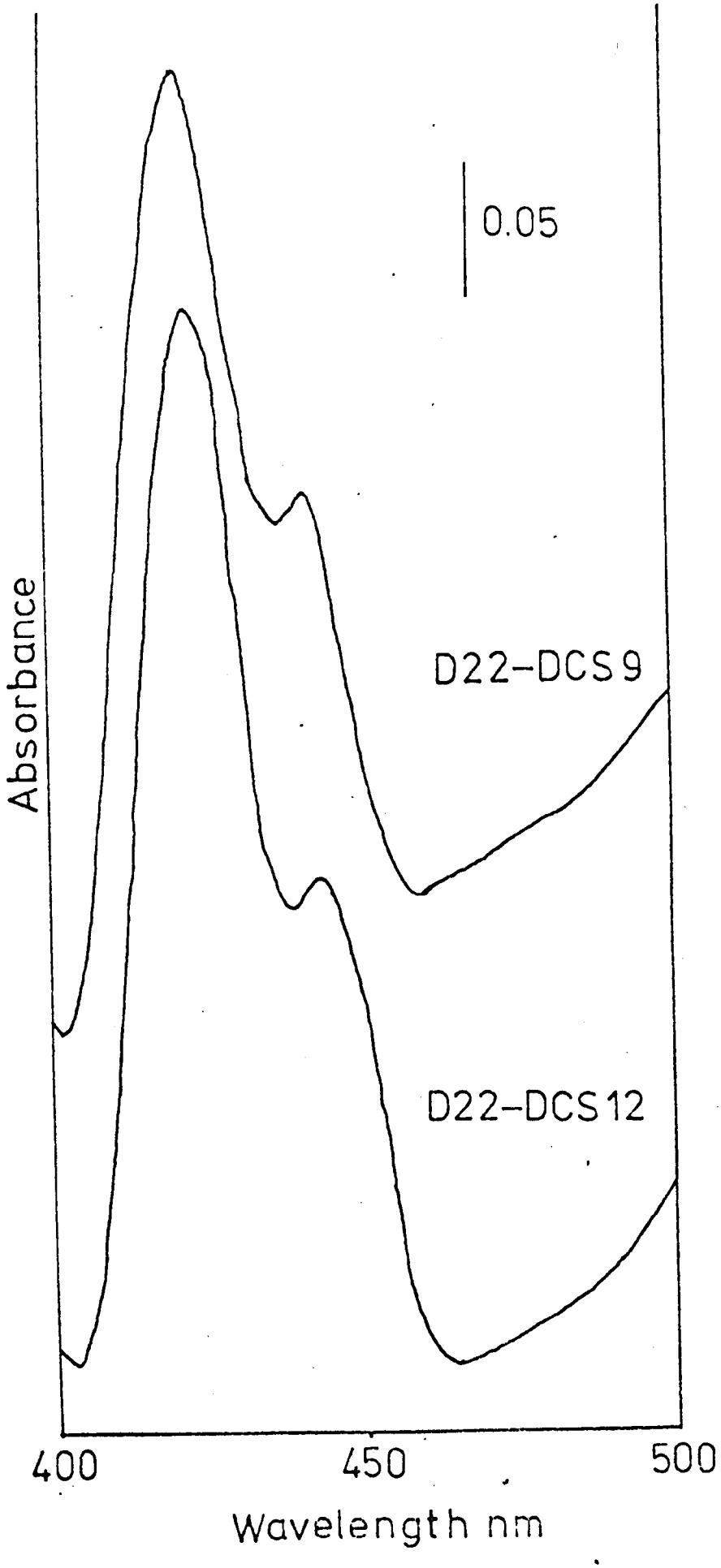


Figure 2.22 B

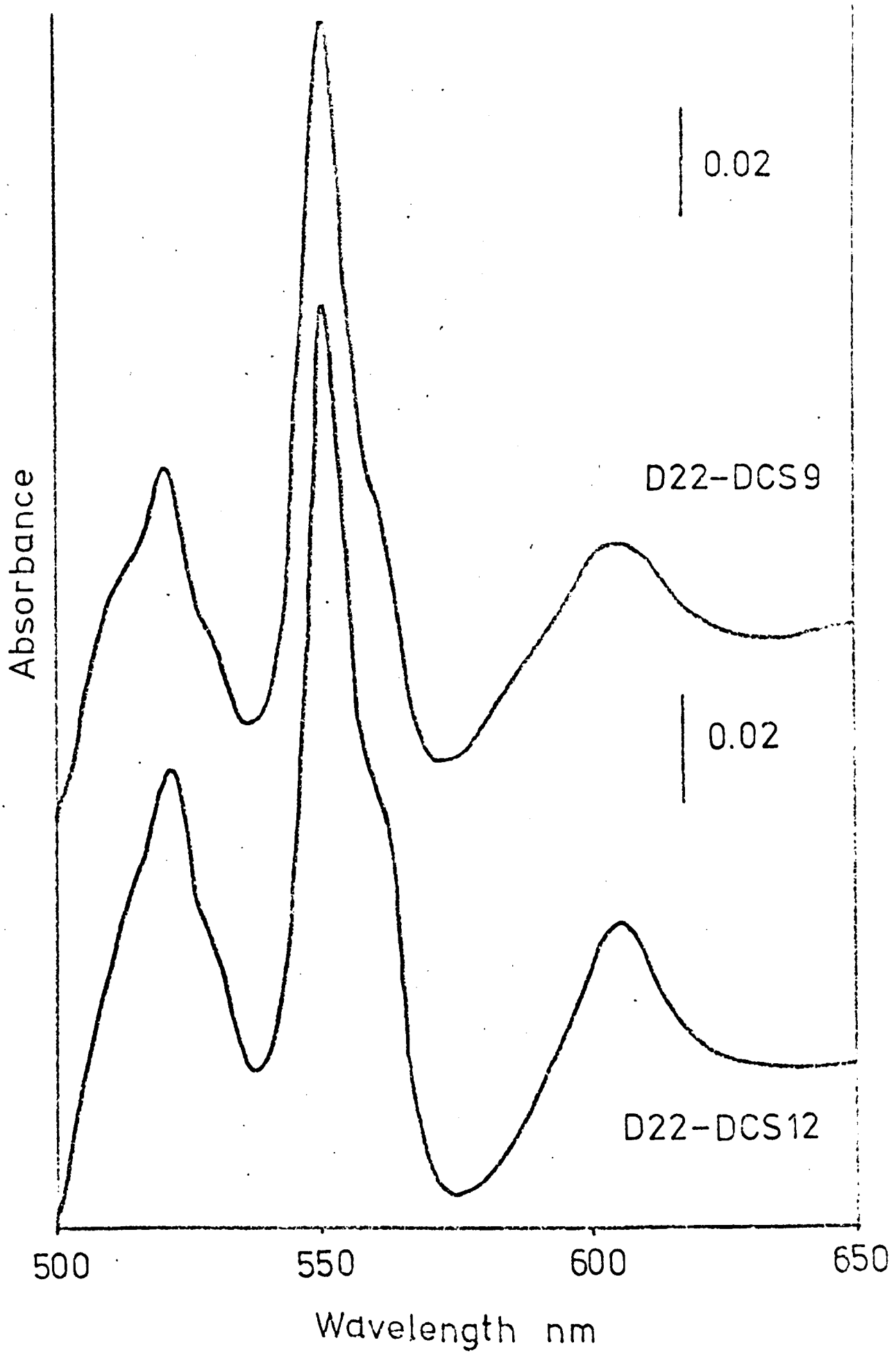

0.02
D22-DCS9
0.02
D22-DCS12
Absorbance
500
550
600
650
Wavelength nm

Figure 2.23A

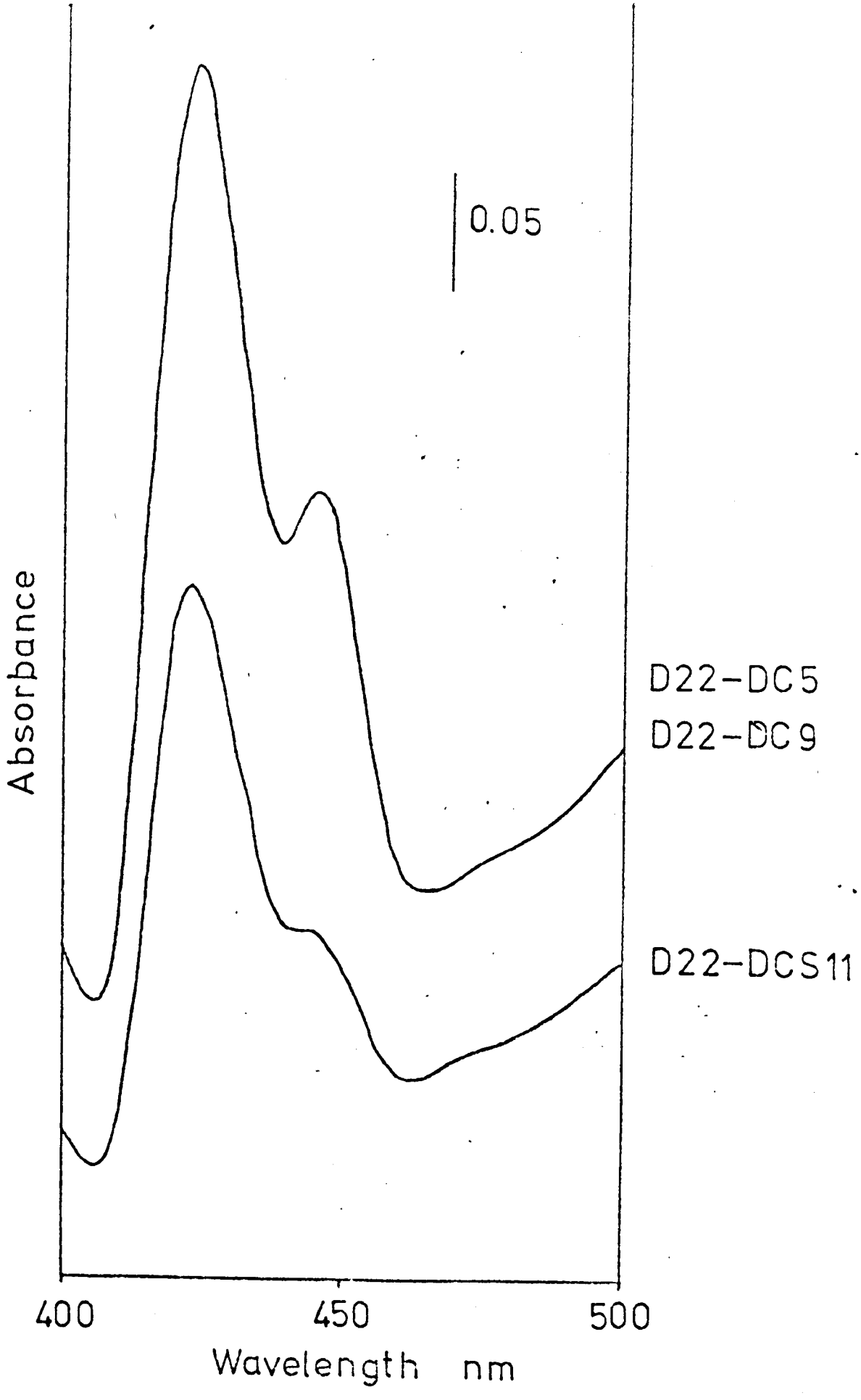

0.05
Absorbance
D22-DC5
D22-DC9
D22-DCS11
400
450
500
Wavelength nm

Figure 2.23B

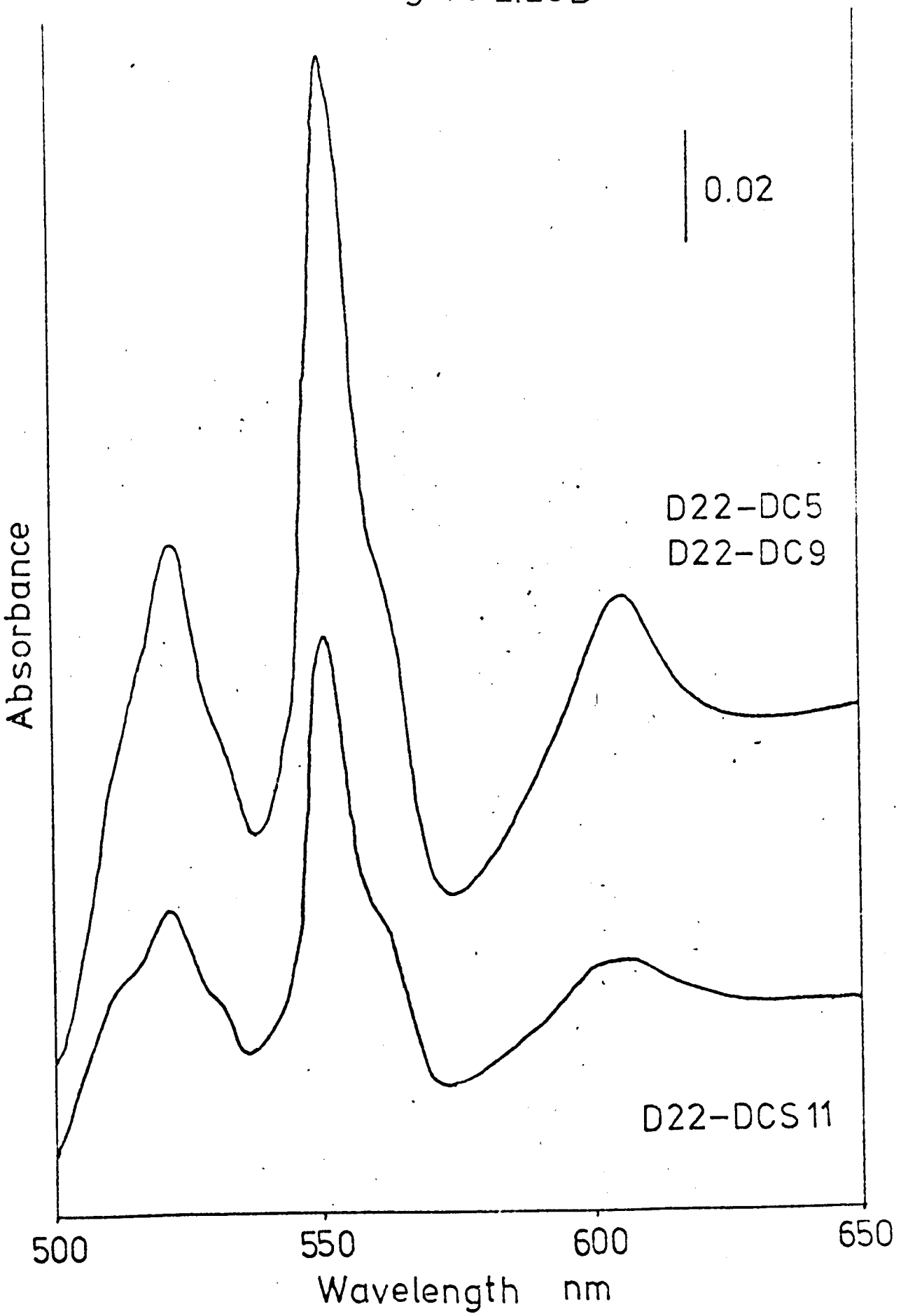

0.02
D22-DC5
D22-DC9
D22-DCS 11
Absorbance
500
550
600
650
Wavelength nm

FIGURE 2.24

Reduced minus oxidised cytochrome difference spectra of S. cerevisiae.
Cells were grown to stationary phase on 0.5% (v/v) ethanol in shake flask culture.
D22- CB9, 57 mg dry weight/ml; D22-CB19, 46 mg dry weight/ml.

FIGURE 2.25

Reduced minus oxidised cytochrome difference spectra of S. cerevisiae.
Cells were grown to stationary phase on 0.5% (v/v) ethanol in shake flask culture.
D22-EC1, 43.5 mg dry weight/ml; D22-EC2, 50 mg dry weight/ml.

Figure 2.24A

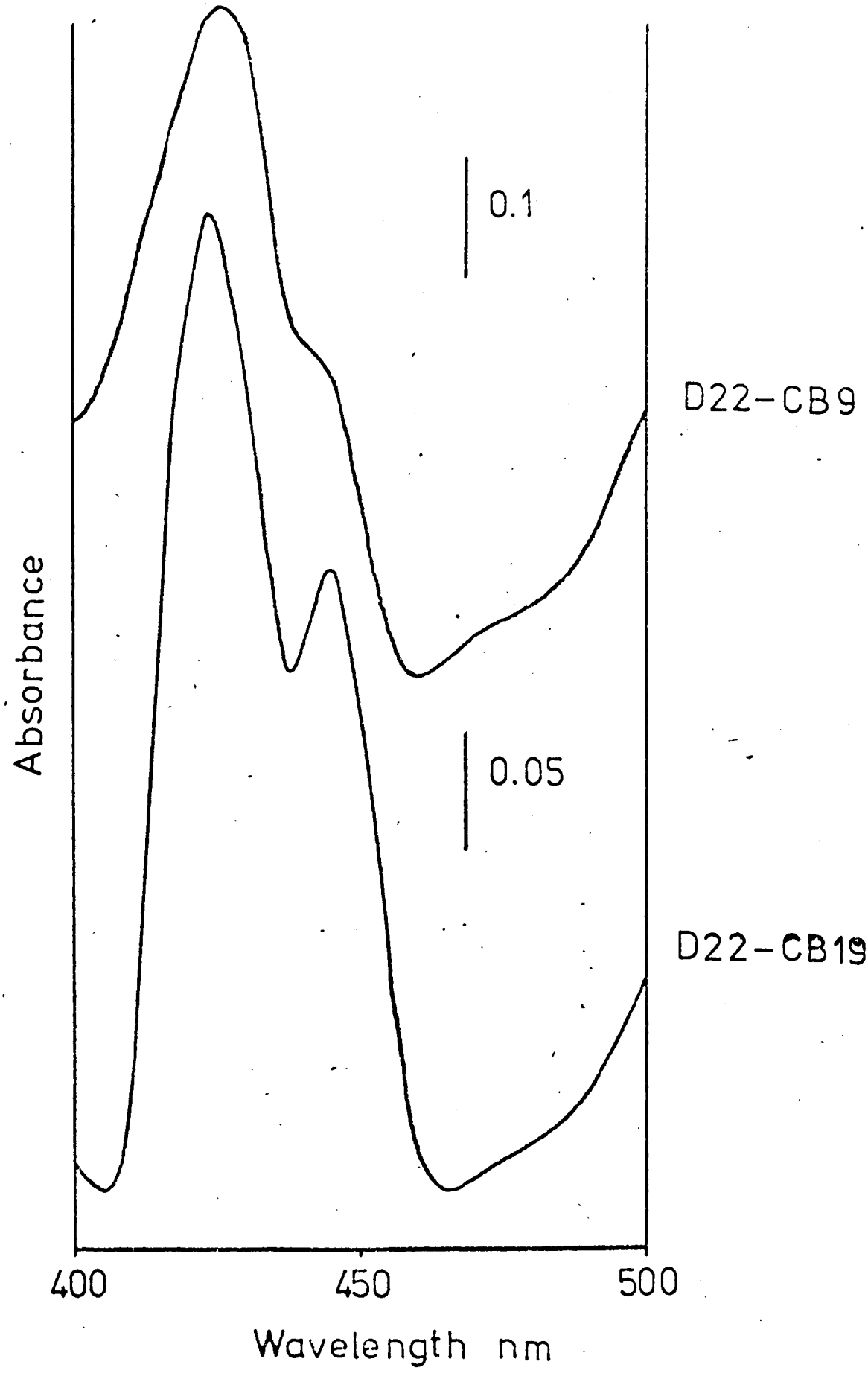


Figure 2.24 B

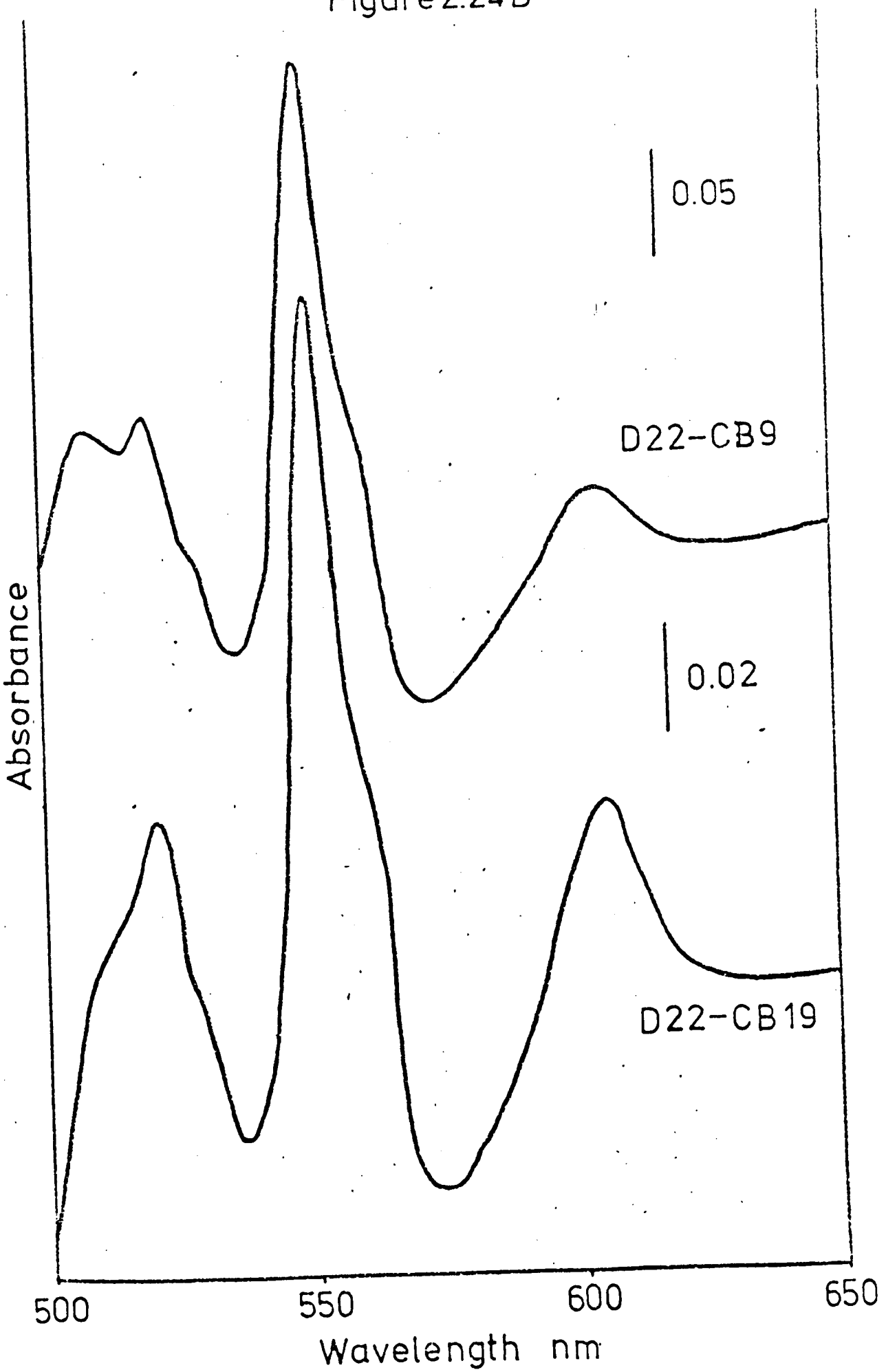

0.05
D22-CB9
0.02
D22-CB19
Absorbance
500
550
600
650
Wavelength nm

Figure 2.25 A

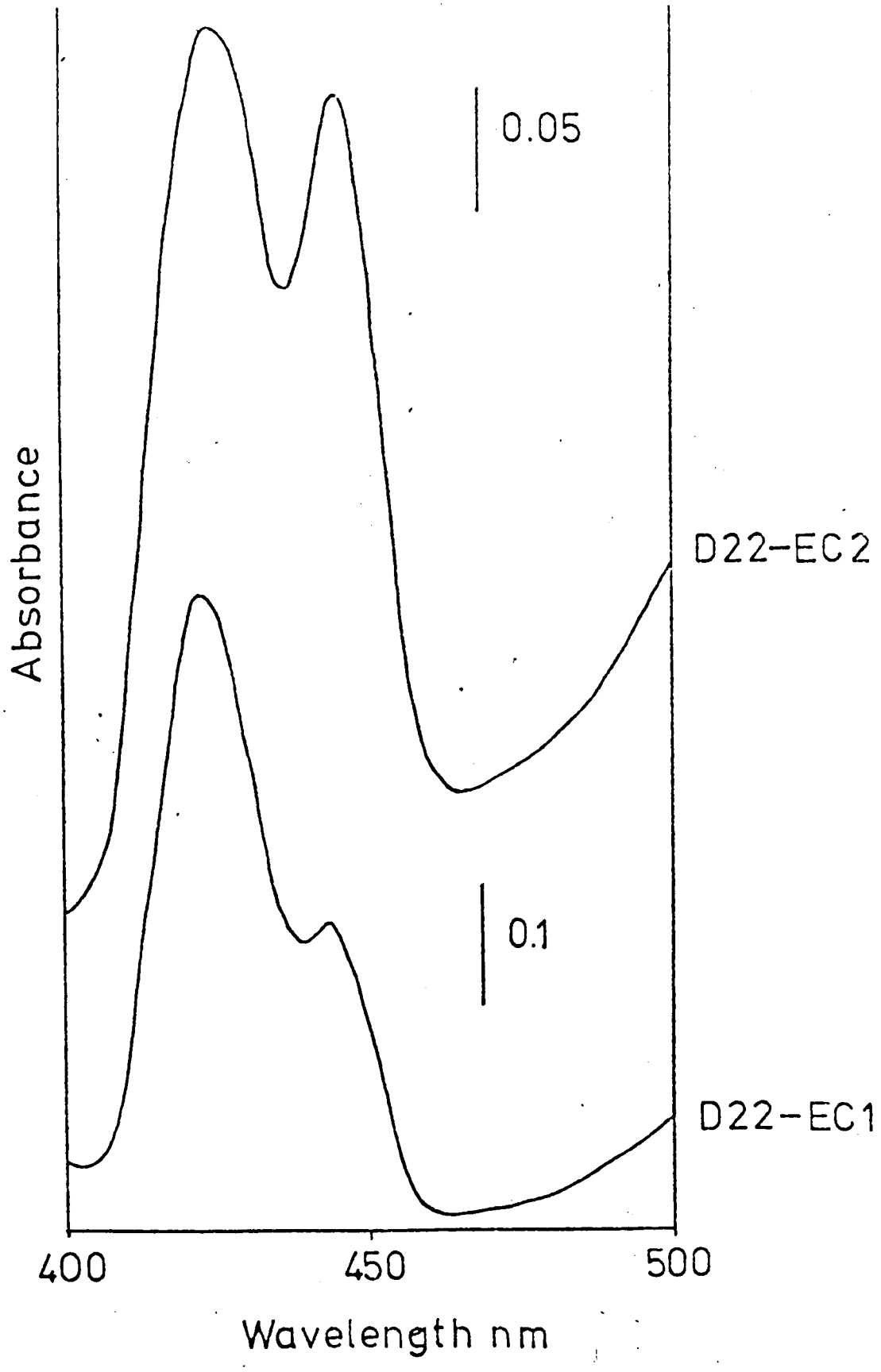


Figure 2.25 B

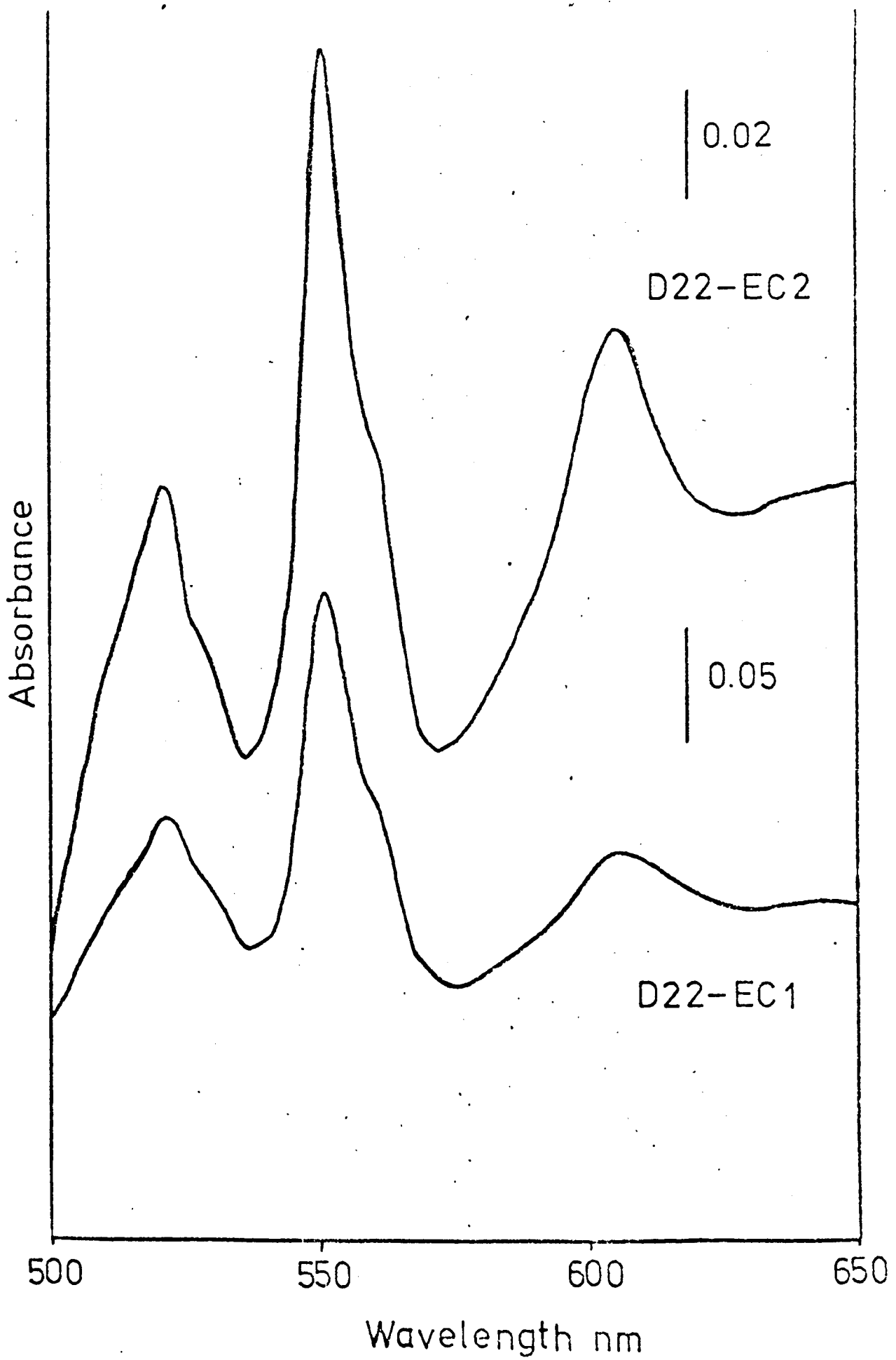

0.02
D22-EC2
0.05
D22-EC1
Absorbance
500
550
600
650
Wavelength nm

TABLE 2.11

Cytochrome contents of S. cerevisiae cells grown on ethanol.

	Growth Conditions	Strain	Type	Class	nmole cytochrome/mg dry weight			Relative Percentage		
					c	b	aa_3	$\frac{c}{c}$	$\frac{b}{c}$	$\frac{aa_3}{c}$
1.	Shake Flask	D22	wild type	-	0.186	0.061	0.046	100	32.8	24.7
2.		D22-DC9	$TTFB^R$	2	0.191	0.046	0.026	100	24.1	13.6
3.		D22-DCS12	$TTFB^R$	2	0.182	0.053	0.03	100	29.1	16.5
4.		D22-DC5	$TTFB^R$	3	0.214	0.059	0.029	100	27.6	13.6
5.		D22-DCS9	$TTFB^R$	3	0.22	0.047	0.024	100	21.4	10.9
6.		D22-DCS11	$TTFB^R$	3	0.195	0.046	0.019	100	23.6	9.7
7.		D22-CB9	"1799"^{R}	1	0.24	0.064	0.022	100	26.7	9.2
8.		D22-CB19	"1799"^{R}	2	0.174	0.059	0.037	100	33.9	21.3
9.		D22-EC1	TET^R	2	0.19	0.058	0.024	100	30.5	12.6
10.		D22-EC2	TET^R	3	0.13	0.046	0.028	100	35.4	21.8
11.	Fermenter	D22	wild type	-	0.168	0.05	0.019	100	29.8	11.3
12.		D22-DCS12	$TTFB^R$	2	0.163	0.048	0.012	100	29.2	7.4
13.		D22-DCS9	$TTFB^R$	3	0.157	0.034	0.01	100	21.6	5.7
14.		D22-CB9	"1799"^{R}	1	0.174	0.047	0.015	100	26.7	8.7
15.		D22-CB19	"1799"^{R}	2	0.16	0.05	0.017	100	31.9	10.6

Notes.

1. Nos. 1 -10 in this Table are analyses of Figures 2.21 - 2.25.

these conditions to early stationary phase on 0.5%(v/v) ethanol is illustrated in Figure 2.21. This may be contrasted with Figure 2.18 (fermenter grown cells). As expected, the most noticeable feature is the large increase in cytochrome $a a_3$ (Table 2.11).

Cytochrome profiles of selected $TTFB^R$ mutants, grown and assayed under the same conditions as the wild type, have also been measured (Figure 2.22, 2.23). The amounts of cytochromes b and c (on a dry weight basis) were in the same range as those of the wild type but in all strains the levels of cytochrome $a a_3$ were less (Table 2.11). Similar results were obtained for the cytochrome contents of "1799"^{R} mutants (Figure 2.24) and of selected TET^R mutants (Figure 2.25). Antimycin was not used in these measurements of cytochrome difference spectra since from previous data (Table 2.10) it was considered legitimate to compare data obtained from reduced minus oxidised spectra alone.

The cytochrome contents of the wild type and various mutant strains grown aerobically on 0.5% (v/v) ethanol in a 10 ltr fermenter to early stationary phase are also shown in Table 2.11. These results are somewhat different to those quoted in Table 2.10 but are most likely due to the different size of fermenter used and to differences in oxygen tension in the culture media. These measurements support the conclusions drawn from Table 2.10. Comparing the cytochrome contents of flask and fermenter grown cells (in Table 2.11) two significant observations are possible. The fermenter grown cells have a lower overall cytochrome content than the corresponding flask grown cells. Second, the wild type has a higher level of cytochrome $a a_3$ than any of the mutants whether flask or fermenter grown. This last point is especially noticeable when relative percentage amounts are compared. While most strains have much less cytochrome $a a_3$, some mutants, eg. D22-CB19 and D22 —EC2, do approach the wild type values whatever the growth conditions.

Cytochrome Contents of S. cerevisiae Mitochondria From Difference Spectroscopy.

S. cerevisiae cells grown to early stationary phase on ethanol in the fermenter were used to prepare mitochondria by the Braun shaker method. These mitochondria were assayed for cytochrome content after further purification on sucrose gradients (Table 2.12). The amounts of cytochromes in these isolated mitochondria may be compared with those of the corresponding

TABLE 2.12

Cytochrome contents of S. cerevisiae mitochondria isolated from ethanol grown cells.

	Growth Conditions	Mitochondrial Preparation	Strain	Type	Class	nmole cytochrome / mg protein.			Relative Percentage		
						c	b	aa_3	$\frac{c}{c}$	$\frac{b}{c}$	$\frac{aa_3}{c}$
1.	Shake flask	Snail Enzyme	D22	wild type	-	1.09	0.59	0.22	100	54.0	24.2
2.	Fermenter	Braun Shaker	D22	wild type	-	1.06	0.55	0.112	100	51.5	10.6
3.	Fermenter	Braun Shaker	D22-DCS12	$TTFB^R$	2	1.12	0.49	0.12	100	43.8	10.7
4.	Fermenter	Braun Shaker	D22-DCS9	$TTFB^R$	3	0.91	0.35	0.117	100	38.2	12.9
5.	Fermenter	Braun Shaker	D22-CB9	"1799"^{R}	1	0.64	0.38	0.09	100	59.5	14.0
6.	Fermenter	Braun Shaker	D22-CB19	"1799"^{R}	2	0.46	0.32	0.076	100	69.1	16.4

intact cells (Table 2.11). For further comparison the cytochrome profile of D22 mitochondria prepared by the Snail Enzyme method (Chapter 3) is included in Table 2.12. These mitochondria were obtained from cells grown in shake flask culture to log phase on 1% v/v ethanol. There were no significant differences between the cytochrome spectra of log and early stationary phase cells grown on ethanol under the same conditions. The results in Table 2.12 are complicated by the variable loss of cytochrome c from the mitochondria during the isolation procedure. The amounts of each cytochrome presented in Table 2.12 are comparable with those reported by Ohnishi et al, (1967) for Saccharomyces carlsbergensis mitochondria. The mitochondria from cells grown in the fermenter; had less cytochrome aa_3 than those from flask grown cells. Mitochondria from $TTFB^R$ strains had levels of cytochrome aa_3 comparable with those of the wild type while the amounts of cytochrome b were somewhat less. For the "1799"^{R} mutants the levels of cytochrome aa_3 were much reduced.

DISCUSSION.

The characteristics of S. cerevisiae mutants, which are resistant to the uncoupler TTFB or to "1799", or to the phosphorylation inhibitor TET have been examined. Measurements of the growth capabilities of selected strains in batch culture on glucose or on ethanol provide an indication that, compared to the wild type, most of the selected mutants are deficient in some aspect(s) of substrate utilisation.

The OL^R mutants and the TET^R mutants that have been isolated in our Laboratory have been shown to be constitutive (Avner and Griffiths, 1970; 1973, a, b; Lancashire and Griffiths, 1971). In the $TTFB^R$ and "1799"^{R} strains examined in this Chapter the resistance phenomenon is always constitutive. Class 2 andClass 3 $TTFB^R$ mutants are supposedly cytoplasmic and are classified as such (Table 2.4), although a contribution from the nuclear genome has not been ruled out completely. This type of mutation to TTFB resistance, and in some cases cytoplasmic mutation to TET resistance, affect the mitochondria so as to produce defective growth on non-fermentable substrate ie. ethanol (Table 2.6). These defects are associated with impaired cellular respiration in representative $TTFB^R$ strains from Class 2 and Class 3 (Figures 2.4 and 2.5). From the nature of the uncoupling agent these deficiencies would be expected to be caused by some primary effect on mitochondrial energy transfer reactions rather than on electron transport or the ATPase complex. On the basis of these Results there is no correlation between the Class of $TTFB^R$ mutant (Table 2.4) and the growth capability on ethanol. However, it is noticeable that the peculiarly defective mutant, D22-DCS11, is temperature sensitive whereas all the others are not.

Mutants selected for resistance to "1799" (Table 2.5) or Class 3, TET^R mutants (Table 2.3) have wild type characteristics for growth on ethanol, in contrast to the Class 3, $TTFB^R$ mutants. This is further evidence for a difference between "1799" and the other uncouplers such as CCCP or TTFB. A similar conclusion has also been inferred from the cross-resistance data presented in Tables 2.2 - 2.5 (see Methods). "1799" may act by a different mechanism to other uncouplers, at a different point in the energy conservation process or at a different site in the mitochondrial membrane. That

inhibitors such as oligomycin or TET have specific binding sites in the inner mitochondrial membrane is well known (Slater and Ter Welle, 1969; Rose and Aldridge, 1969) but the existence of similar sites of interaction with uncouplers has only recently been directly shown (Hanstein and Hatefi, 1974 a, b). The operation of the same pattern of crossresistance for all the mutants so far obtained in our Laboratory (Tables 2. 2 - 2.5) would also indicate a similar mechanism of resistance, and therefore of interaction, at the mitochondrial level for all the agents used. Since some TET^R mutants are also cross-resistant to "1799", but not to other uncouplers, this may indicate that "1799" may have other effects in addition to its uncoupling activity.

Equally well, it is possible that resistance to TET may arise in other areas of the mitochondrial membrane in addition to the ATPase complex. These may or may not result from the same single gene, cytoplasmic mutation. Tri-alkyl tin compounds are inhibitors of oxidative phosphorylation (Aldridge and Street, 1970) but also catalyse the transport of various anions, eg. halides, across the inner mitochondrial membrane (Selwyn et al, 1970; Stockdale et al, 1970; Rose and Aldridge, 1972). Expression of these effects is modified by pH (Coleman and Palmer, 1971). TET does have some uncoupling activity, but in any case by catalysing anion permeation it would act to dissipate the energy conserved in electron transport away from ATP synthesis. In addition tri-alkyl tin compounds may also inhibit the adenine nucleotide translocase of the mitochondrial inner membrane (Harris et al, 1973). Yeast mutants, resistant to TET, may conceivably show resistance at the mitochondrial level to any of these effects, In this context it is interesting that Class 3, TET^R mutants, which are cross-resistant to "1799" (Table 2. 3) are also resistant to bongkrekic acid (Cain et al, 1974). This also is an inhibitor of the mitochondrial adenine nucleotide translocase (Henderson and Lardy, 1970).

The majority of the mutants that were tested were defective in growth on glucose, relative to the wild type. In Saccharomyces sp. the formation of the mitochondrial enzymes concerned with oxidative phosphorylation is inhibited by high concentrations of glucose, even in aerobic culture (Jayaraman et al, 1966). This substrate supports a high rate of growth by fermentation but substrates incapable of supporting such a high rate cause less marked repression (Beck and von Meyerburg, 1968). As expected, most of the selected S. cerevisiae mutants that were examined were unaffected in the early, fermentative phase of growth on glucose. As the concentration of glucose in the culture medium falls the yeast undergo a period of respiratory adaption in order to utilise the ethanol

produced. This causes the appearance of a definite inflection in the growth curve. At very low concentrations of glucose, repression cannot be observed in this fashion. Such is the case with S. cerevisiae, strain D22, grown aerobically on 0.4% (w/v) glucose. Under these conditions, inflections in the growth curves of the various resistant mutants may indicate that the mutants are more amenable to glucose repression (Figures 2.8 and 2.9). In the case of the $TTFB^R$ strains, since the oxidative phosphorylation system is defective, fermentative growth would be more preferred under the initial conditions. The mitochondria would be expected to deteriorate and development to support growth on ethanol would give rise to a short lag period. In contrast to the results on ethanol, the "1799"^{R} mutants were defective in growth on glucose. Although the operation of mitochondrial oxidative phosphorylation may be unaltered with respect to the wild type, it is possible in this case that there are deficiencies in the biogenesis of functional mitochondria. It is interesting that the Class 3, TET^R strain, D22 - EC2 should have the same growth characteristics on ethanol and glucose as the wild type. This would have more in common with the OL^R mutants (Avner and Griffiths, 1973a) and may be the result of a much more localised change in the mitochondrial membrane than would be expected from an examination of its cross-resistance profile (Tables 2.2 and 2.3).

Groot et al, (1971) have shown that promitochondria present in glucose repressed S. cerevisiae cells are able to catalyse uncoupler sensitive, energy transfer reactions even in the absence of a respiratory chain. The $TTFB^R$ mutant, D22 - DCS11, is defective in fermentative growth on glucose. It is feasible that the mutation to uncoupler resistance in this strain has affected these residual energy transfer reactions and that this may be a characteristic of temperature sensitive $TTFB^R$ mutants.

These growth curves provide further evidence for the involvement of a mitochondrial factor in uncoupler or TET resistance. In contrast to the OL^R mutants (Avner and Griffiths, 1973 a, b) this factor may be associated with defects in the oxidative phosphorylation process appearing as a result of the mutation.

It is of interest to know whether a mutant has the same growth yield as the wild type even if it grows at a lower rate and has a lower rate of respiration. It would then be possible to infer that the efficiency of oxidative phosphorylation in the mutant is the same as the wild type but that the mutation has resulted in the insertion of a rate limiting step at the mitochondrial level. If the mutant

has a lower yield then it is possible that the efficiency of oxidative phosphorylation is also lowered. From the initial growth curves on ethanol only the "1799"^{R} strains and the TET^{R} mutant, D22-EC2 have the same growth rates as the wild type (Table 2.6); and only these, plus the TTFB^{R} strain D22 - DCS12, attain the same cell concentration as the wild type in stationary phase (Figures 2.1 - 2.3).

Detailed studies of molar growth yields on ethanol show that only the mutants D22 - CB9 and D22 - DCS12 reach the same cell concentration as the wild type in batch culture (Figure 2.10). These results are somewhat at variance with the initial data on the strains D22 - CB19 and D22 - EC2. However, these differences are probably due to errors in the measurements of the growth curves on ethanol. On more detailed examination both these strains have molar growth yields approaching the wild type (Table 2.7). Even if a mutant has a lower respiratory rate and a lower growth rate than the wild type, it may still reach the same population levels although after a longer time, ie. D22 - DCS12. On glucose, none of the mutants examined had the same molar growth yield as the wild type (Table 2.8) although, in line with the initial data obtained from growth curves, D22 - EC2 made the closest approach.

Avner and Griffiths, (1973a) have estimated the growth yields of Class 2, OL^{R} mutants and found them to be unchanged from the wild type. The yields of the Class 1 mutants were however 15-20% less on ethanol. The molar yields quoted by Avner and Griffiths, (1973a) were much less than the corresponding values shown in these Results, which are comparable with those obtained by Kormancikova et al, (1969). This discrepancy is most likely due to differences in the makeup of the culture media.

The molar growth yield of any microorganism not only depends on the efficiency of energy conversion but also on the amount of substrate assimilated into cellular material. It is also affected by the proportion of substrate expended as maintainence energy and the amount of energy required for unit cell growth. Only if these other factors remain constant can alterations in the growth yield be equated with alterations in the efficiency of the energy conservation reactions. The use of batch culture methods for the calculation of growth yields may allow the inclusion of systematic errors since the ATP coefficient (Y_{ATP}) for any organism may be affected by the cell composition, the specific growth rate and the maintainence coefficient. The maintainence

coefficient is that amount of substrate required to produce energy for maintainence per unit amount of cells per unit time, and at higher growth rates this decreases (Stouthamer and Bettenhausen, 1973). This is in contrast to previous conclusions that Y_{ATP} is a constant for different microorganisms (Bauchop and Elsden, 1960; Senez, 1962; Forrest and Walker, 1971). Recent experiments have shown that Y_{ATP} values may vary for different organisms (de Vries et al, 1970; McGill and Davies, 1971) but that the Y^{max}_{ATP} (molar yield per mole ATP corrected for maintainence energy) stays constant. The lower the specific growth rate the lower the Y_{ATP} and this is true for any value of the maintainence coefficient. In Tables 2.7 and 2.8 the maintainence energies are assumed to be the same in every case and the slower growing mutants still have lower Y_{ATP} values. If the higher maintainence coefficients for the mutant strains are taken into account the corresponding Y_{ATP} values are lower than that of the wild type but the efficiency of oxidative phosphorylation may then be presumed to be the same in all strains. It is possible to differentiate between these two possibilities by using continuous culture methods.

No mutant was found which showed a higher growth yield than the wild type on either fermentative or non-fermentative substrate in batch culture. Indeed most were only able to achieve much lower values. It is not surprising that increases in growth yield were not seen since evolutionary theory provides a powerful argument against such an occurrence. It is therefore presumed that a lower growth yield is due primarily to a deficiency in the oxidative phosphorylation system of the mutant, associated with the particular resistance mutation.

The Class 2 and Class 3, uncoupler or TET resistant, mutants that have been examined have, in terms of growth yields, more in common with Class 1, OL^R strains than with the cytoplasmic, Class 2, OL^R types (Avner and Griffiths, 1973a). Therefore resistance to these agents, arising by cytoplasmic mutation, may be expressed in a more widespread effect on the properties of the inner mitochondrial membrane. The strains tested for growth yields fall into two groups on both glucose and ethanol (Tables 2.7 and 2.8) so it may be argued that some cytoplasmic mutations to uncoupler or TET resistance result in more widespread effects than others. The only correlation is that mutants resistant or cross-resistant to "1799" approach wild type values. In this respect the classification of D22 DCS12 as Class 2, $TTFB^R$ (Griffiths, 1972) may not be correct as it is resistant to "1799" at the cellular and mitochondrial levels (see Chapter 3).

A decrease in the rate of respiration of the intact cell can be caused either by some defect in the mitochondria such that the rate of oxygen uptake is less and the number in the cell in unchanged, or the number of mitochondria in the cell may be lowered. This second possibility can be discounted by electron microscopy (P. R. Avner, 1973). The fact that mitochondria from $TTFB^R$, and TET^R mutants do posses defects in cytochrome content and in their capability for oxidative phosphorylation (Chapter 3) is evidence in favour of the first possibility.

The cytochrome profiles of S. cerevisiae, strain D22, and of selected TET or uncoupler resistant mutants show only quantitative differences. The wild type strain is capable of synthesising a higher content of cytochrome $a\,a_3$ than any of the mutant strains grown under the same conditions (Table 2.11). In all cases the cells were cultivated in the dark to avoid any effect of light on the cytochrome content (Ninneman et al, 1970). It is possible to correlate variations in cytochrome $a\,a_3$ levels with the growth characteristics of the different strains in aerobic, batch culture on glucose or on ethanol. A decrease in the content of functional cytochrome $a\,a_3$ is generally reflected in the growth yield of the mutant compared to the wild type (Tables 2.7 and 2.8). The mutants D22 - CB19, D22 - DCS12 and D22 - EC2 approach the wild type in terms of growth yields and also in cytochrome $a\,a_3$ content. In contrast, the "1799"^{R} strain, D22 - CB9 has a relatively low cytochrome $a\,a_3$ level in the cell but is very similar to the wild type in growth characteristics. D22 -CB9 is a Class 1 mutant (Table 2.5) and from comparison with previous data (Avner and Griffiths, 1973a) would be expected to have inferior growth characteristics. Variations in the cytochrome contents of the cell, which would be expected in all types of Class 1 mutants (Griffiths, 1972) are also characteristic of Class 2 and Class 3 uncoupler or TET resistant strains. These differences are expressed as changes in the amount of cytochrome $a\,a_3$ in the mutant cells with the other cytochromes largely unaffected. On the other hand Class 2, OL^R mutants, eg. D22 - A16, have the same growth characteristics as the wild type (Avner and Griffiths, 1973a) and essentially unchanged cytochrome contents.

Experiments utilising potentiometric titration show that there are several chemically distinct species of b-type cytochromes in mitochondria from rat liver (Wilson and Dutton, 1970) pigeon heart (Chance et al, 1970) and the yeast Candida utilis (Sato et al, 1972). In C. utilis, one has a double α-band at 565 nm and at 558 nm (562 nm and 555 nm at 77° K) and is similar to the cytochrome b_T observed in pigeon heart mitochondria (Sato et al, 1971a).

The second has a single α-band at 561.5 nm (558 nm at 77°K) and is identified with cytochrome b_K (Sato et al, 1971a). The third has an α-band at 563 nm (561.5nm at 77°K) and has not been further characterised.

According to Figure 2.15, S. cerevisiae cells at 77°K have a single absorption band in the cytochrome b region at 558 nm. This is identified as cytochrome b_K. There are shoulders at 553 nm and at 548 nm, and it is probable that these absorption bands are due mostly to contributions from cytochromes c and c_1. There is no evidence for absorption bands characteristic of cytochrome b_T.

The cytochromes present in the respiratory chain (b_T, b_K, c_1, c, a and a_3) have corresponding half-reduction potentials of -30 mV, +30 mV, +215 mV, +235 mV, +210 mV and +385 mV respectively. It is possible to show by measurements in uncoupled mitochondria versus those under a high phosphate potential that shifts in the mid-point potentials of certain cytochromes occur (Wilson and Dutton, 1970b). Some components of the respiratory chain, eg. cytochrome b_K, are relatively unchanged between these two states, while other cytochromes undergo relatively small shifts in half-reduction potential in the presence of ATP; cytochrome c going from 215 mV to 175mV and cytochrome a changing from 210mV to 265 mV. By contrast, ATP induces a change in the mid-point potential of cytochrome b_T from -30 mV to +245 mV while that of cytochrome a_3 changes from +385 mV (uncoupled) to +155 mV (Wilson et al, 1972; Wilson and Erecinska, 1972).

When recording reduced minus oxidised cytochrome difference spectra of intact S. cerevisiae cells, uncoupler may be added to the oxidised sample in order to collapse the phosphate potential and ensure that cytochrome a_3 is more completely oxidised.

Use of antimycin can visualise the absorption bands due to b-type cytochromes alone (Figures 2.15 and 2.19). Antimycin addition completely reduces these cytochromes in the aerobic state. Subsequent to this, removal of oxygen by dithionite addition causes partial oxidation (Chance, 1952; Kovac et al, 1970; Sato et al, 1972). Antimycin added to cells that are initially anaerobic will, however, also induce the oxidation of cytochrome b_T. In these experiments, antimycin was added under anaerobic conditions, and so the absorption band due to this cytochrome would not be expected to be large (Figure 2.15).

Antimycin is also useful in the measurement of cytochromes c and aa_3 (Tables 2. 9 and 2. 10) because not only does it remove the contributions of the b-type cytochromes from the spectrum (Figures 2. 16 and 2. 20) but it also allows a greater net oxidation of cytochromes c and aa_3 in the reference (oxidised) cell as it stops electron transport. The susceptibility of cytochrome a_3 to oxidation is also enhanced because of the collapse in phosphate potential associated with antimycin addition. In most cases the yeast present in the sample cell were reduced by addition of dithionite which causes maximal reduction of all cytochromes.

Several different types of mutation give rise to variations in the cytochrome contents of yeast cells. The "petite" mutation in Saccharomyces sp. has been well characterised and results in a gross change in mitochondrial structure such that the cell is unable to utilise non-fermentable substrates for growth. The cytochromes c_1, b, a and a_3 are completely lacking from such mitochondria. This phenotype can also arise by chromosomal rather than mitochondrial mutation. Single gene, nuclear mutants having a variety of deficiencies in energy metabolism have been isolated and some strains are characterised by a partial or complete lack of one or more cytochromes (Sherman, 1964; Sherman and Slonimski, 1964; Reilly and Sherman, 1965). Mutant strains have also been derived with more than one single gene chromosomal mutation (Subik et al, 1970; Subik et al, 1972a) or with a chromosomal plus a "petite" mutation (Kovacova et al, 1968). Other strains of yeast having defects in the tricarboxylic acid cycle and in cytochrome biosynthesis have been reported (Ogur et al, 1965; Subik et al, 1972a).

The first large group of single gene, nuclear, cytochrome deficient mutants consists of those which are unable to grow on non-fermentable substrates and have only cytochrome c and may also possess a "petite" cytoplasmic geno-type (Beck et al, 1971). These include several of the series of "p" mutants, described by Sherman, (1963, 1964, 1967) and other workers (Sherman and Slonimski, 1964; Mackler et al, 1965; Hawthorne and Mortimer, 1968). The second class contains chromosomal "p" mutants with alterations in the level of one or two cytochromes only (Sherman and Slonimski, 1964) and also the mutants "cy_1 - cy_6" (Reilly and Sherman, 1965). The "cy_1" mutation leads to a deficiency in the content of cytochrome c (Mattoon and Sherman, 1966) and this gene codes for the primary structure of iso-1-cytochrome c in S. cerevisiae (Sherman et al, 1968).

Two other groups of single gene nuclear mutants are of interest in this context. There are some mutants of Saccharomyces sp. which have no measurable cytochrome deficiency but which are unable to grow on non-fermentable substrates eg. the strains "p_9" or "op_1" (Kovac et al, 1967a; Beck, et al, 1968), "aem_{1-11}" (Parker and Mattoon, 1969) and "glt_1" (Ogur et al, 1964). These strains have the phenotype of an oxidative phosphorylation deficient mutant. Certain nuclear mutants have also been isolated which are resistant to antibiotics and other agents specifically affecting mitochondrial functions. Strains of Saccharomyces yeast have been selected for resistance to oligomycin (Parker et al, 1968) or to the uncoupler DNP (Parker and Mattoon, 1968). Nuclear mutants of Candida utilis having resistance to antimycin have also been reported (Butow and Zeydel, 1968; Grimmelikhuyzen and Slater, 1973). More recently chromosomal mutants of S. cerevisiae resistant to inhibitors of mitochondrial membrane transport processes have been described (Lauquin et al, 1973; Perkins et al, 1973).

Of the strains selected for examination in this Thesis, all are cytoplasmic mutants except for the "1799"^{R} strain, D22 - CB9 (Tables 2.1 - 2.5). Class 1 mutants, which involve both the nuclear and mitochondrial genetic systems are "general membrane mutants" having resistance to many different mitochondrial inhibitors (Griffiths, 1972; Avner and Griffiths, 1973a, b). It is supposed that the widespread effects on the properties of the inner mitochondrial membrane are due to the presence of simultaneous mutation(s) in either genome. Assuming that Class 2 and Class 3 mutants arise by alteration of a single cytoplasmic gene, the expected result would be a single change in a mitochondrially synthesised membrane subunit. This has not yet been shown experimentally. However, mitochondrial DNA can direct the synthesis of a membrance component which influences the activity of the mitochondrial ATPase in cytoplasmic OL^R mutants of S. cerevisiae (Shannon et al, 1973). There is no reason why a single gene, cytoplasmic mutation should not give rise to more general effects leading to defects in oxidative phosphorylation. In addition, for the Class 2 and Class 3, $TTFB^R$ strains, more than one cytoplasmic gene may be involved in the mutation and some contribution from the nuclear genetic system has not been completely discounted.

Assuming that a single gene mutation results in a change in a single component of the mitochondrial membrane, Changeux and Thiery, (1968) have discussed the importance of the cooperative effects in mitochondrial membranes.

On this basis a change in only one subunit could affect the entire membrane complex and this may result in an overall defect in oxidative phosphorylation. Similarly, the action of a particular inhibitor, which binds a specific site, may give rise to more widespread changes in the structure of the membrane through the existence of cooperative interactions. It has been proposed that mitochondrial ribosomes are themselves integrated with the mitochondrial membrane (Dixon et al, 1974), in line with observations on cytoplasmic species (Glazer and Satorelli, 1972). Protein synthesis inhibitors might then express secondary effects, eg. inhibition of respiration (Firkin and Linnane, 1968), through the integrated complex. Inhibitors or uncouplers of oxidative phosphorylation can affect mitochondrial protein synthesis by interrupting the supply of ATP. There is also the possibility that they may produce direct effects on mitochondrial ribosomes by cooperative effects. In contrast there is evidence that a single gene, cytoplasmic mutation to antibiotic resistance, presumably giving rise to a single change at a particular site in the mitochondrial membrane, may leave other membrane functions relatively unaffected (Avner and Griffiths, 1973a, b).

In conclusion, mutation to TTFB resistance may primarily affect uncoupler binding sites in the mitochondria (Hanstein and Hatefi, 1974a, b) and as a result give rise to a widespread change in membrane properties resulting in deficiencies in growth and respiration on non-fermentable substrates. The differences in cytochrome aa_3 contents of the mutants, relative to the wild type, may or may not be a direct cause of lower growth rates and/or yields. Resistance to "1799" arises in a different fashion to $TTFB^R$, and "1799"^{R} mutants more closely approach wild type characteristics. TET has several effects at the mitochondrial level and it is possible that some of these may also be associated with "1799". As a result there may be different types of TET^R mutant which may approach wild type characteristics in growth and cytochrome content to varying degrees.

CHAPTER 3. Effects of Inhibitors or Uncouplers on Respiration in Cells and Mitochondria, and on the Mitochondrial ATPase of Saccharomyces cerevisiae.

INTRODUCTION

The selection of mutants of S. cerevisiae resistant to uncouplers or to inhibitors of oxidative phosphorylation was accomplished in our Laboratory using plating techniques (Griffiths, 1972). Resistance to any of these agents may arise in several ways, and resistance may or may not be expressed at the mitochondrial level. A change in the permeability properties of the cell membrane may occur such that the inhibitor or uncoupler is prevented from reaching its site of action. Equally, some "detoxification" mechanism could arise such that the agent is itself rendered ineffective. However these possibilities are unlikely in view of the well defined mitochondrial genetics of the mutants used in this study (Griffiths, 1972).

Although inhibitors and uncouplers of oxidative phosphorylation each have a well defined site of action at the mitochondrial inner membrane some of these agents may affect the permeability properties of the cell membrane. These would have adverse effects on metabolism and in Class 1 mutants it is possible that resistance may be manifested at this level. In cells sensitive to the effects of inhibitors or uncouplers on mitochondrial oxidative phosphorylation it is presumed that they die owing to lack of ATP. However in the long term, death may be produced by the disintegration, by the uncoupler, of essential mitochondrial membrane complexes (Towers et al, 1973).

Some of the experiments in this Chapter were performed in order to provide information on the immediate effects of TET or uncoupling agents on intact cells. Measurements of the cellular rate of oxygen uptake complement those in Chapter 2 and uncouplers or TET have immediate effects on this rate. The respiratory activities and phosphorylation efficiencies of isolated mitochondria from S. cerevisiae, D22 and mutant strains have been examined.

These data provide some correlation with the growth yields of the corresponding strains shown in Chapter 2. Resistance to uncouplers or to TET has been established to be at the mitochondrial level by measurements of the effects of these agents on mitochondrial oxygen uptake. These observations are in line with the indications of genetic analyses (Griffiths, 1972).

The mitochondrial Mg^{2+} - dependent ATPase activity is a partial reaction of oxidative phosphorylation (Myers and Slater, 1957); and it is sensitive to such inhibitors as oligomycin (Lardy et al, 1958; Slater and Ter Welle, 1969); DCCD (Beechey et al, 1967; Roberton et al, 1968) or TET (Aldridge and Street, 1964; Stockdale et al, 1970).

Recent approaches to the resolution of energy conservation reactions have been through the use of micro-organisms which have a defective ATPase activity as a result of mutations (Sato et al, 1971b; Butlin et al, 1971, 1973; Abrams et al, 1972; Nieuwenhuis et al, 1973, Cox et al, 1974). In S. cerevisiae the effects of the cytoplasmic "petite mutation" on the mitochondrial ATPase have been described (Kovac and Weissova, 1968; Schatz, 1968; Schatz and Criddle, 1969). The development of the ATPase activity during mitochondrial biogenesis is also relevant in this context (Criddle and Schatz, 1969; Paltauf and Schatz, 1969; Watson et al, 1971). The kinetic properties of the Mg^{2+} - dependent ATPase of S. cerevisiae mitochondria were initially studied by Kovac et al, (1968) and by Somlo, (1968). A comparison with the wild type, of the properties of the ATPase present in selected uncoupler or TET resistant mutants of strain D22 has been made. Resistance to TET is shown at the mitochondrial level and the mutations to uncoupler resistance also produce some decrease in the sensitivity of the ATPase activity towards oligomycin. Resistance to oligomycin is seen in several series of nuclear and cytoplasmic yeast mutants that have been obtained by other workers (Parker et al, 1968; Avner and Griffiths, 1970; Goffeau et al, 1970; Stuart, 1970; Wakabayashi and Gunge, 1970; Mitchell et al, 1972).

METHODS AND MATERIALS

Growth of S. cerevisiae.

The cells were grown in 500 ml medium in 2 ltr conical flasks which were baffled for efficient aeration. The medium contained 0.5% (w/v) yeast extract; mineral salts according to Wickerham, (1946) and described in Chapter 2, and 1.0 g adenine sulphate per litre of distilled water. The pH was adjusted to 5.5 and the substrate was 0.5% (v/v) ethanol. The inoculum was a 1.0% (v/v) sample of a starter culture grown to early stationary phase on ethanol (0.5% v/v). The cultures were grown in the dark at 30°C in a Gallenkamp Rotary Incubator at 200-300 rpm.

Yeast were also grown in 10 ltr cultures inNew Brunswick fermenters. The composition of the medium was as above and the carbon scource was ethanol. Tributyl citrate was used as antifoaming agent at a concentration of 0.1 - 0.2 ml/ltr. The yeast were grown in the dark at 30°C with stirring at 300-500 rpm. Air was bubbled through the medium at the rate of 1 ltr air/ ltr medium/min. A 1.0%(v/v) inoculum was used as described above. For growth of the culture to early stationary phase the ethanol concentration was 0.5% (v/v) but for growth to log phase the concentration was 1.0% (v/v).

Preparation of Mitochondria (Snail enzyme).

Cells grown in the fermenter were harvested in the logarithmic phase of growth, using an MSE "Mistral" centrifuge, and washed with distilled water. These and all subsequent centrifugations were done between 0°C and 5°C. The cells were further washed with buffer containing 10mM EDTA, 100 mM Tris-HCl, pH9.3. After centrifugation the cells were resuspended in this buffer in the ratio cell wet weight: buffer volume = 1: 2.5. Mercaptoethanol was added, in the proportion of 0.1 ml per 10 g wet weight cells, and the suspension incubated in a Gallenkamp rotary incubator at 30°C and 100-150 rpm for 30 min.

The cells were then spun down at 4000 g for 2 min and resuspended in buffer containing 1M Sorbitol, 2mM EDTA, 50 mM citrate-phosphate, pH5.8. [All sorbitol solutions used for making buffers were first deionised by stirring with "Dowex 50" (H^+ form) ion exchange resin for 2 hr (Kovac et al, 1972).] The ratio of cell wet weight: buffer volume = 1: 1.5. Snail enzyme ("Helicase") was added to this cell suspension in the quantity of 1ml enzyme : 10g wet weight cells and the mixture incubated at 30°C in a Gallenkamp rotary incubator at 100 rpm. The digestion of the yeast cell wall was followed by diluting a 0.1 ml aliquot of this suspension into 10 ml distilled water and reading the turbidity on a Bausch and Lomb "Spectronic 20" spectrophotometer at 600 nm. After approximately 1 hr, when the turbidity readings had decreased by 30-50%, the suspension was centrifuged at 4000 g, for 5 min. and the protoplasts washed twice with buffer containing 1M Sorbitol, 2 mM EDTA, 20 mM Tris-HCl, pH 7.4. They were then resuspended in buffer, consisting of 0.5M Sorbitol, 2mM EDTA, 20 mM Tris-HCl, 0.2% (w/v) Bovine Serum Albumin (Fraction 5), pH 7.4, in the proportions of 1 g wet weight cells: 2.5 ml buffer. This suspension was then gently homogenised in a hand homogeniser. Since S. cerevisiae, strain D22, is relatively resistant to snail enzyme treatment this suspension was then put through a French Press at 0°C and at 1-2000 lbs/sq. in.

In order to separate unbroken cells and protoplasts the homogenate was centrifuged at 650 g for 15 min, keeping the supernatant. This fraction was subsequently centrifuged for 15 min at 1000 g and then for 15 min at 1500 g, always retaining the supernatant. To obtain the mitochondria the final supernatant was centrifuged at 20,000 g for 40 min. The mitochondrial pellet was resuspended in buffer (0.5 M Sorbitol, 1 mM EDTA, 10 mM Tris-maleate, pH 6.5) to a protein concentration of about 15 mg/ml and stored at 0°C. The yield was usually at least 1 mg mitochondrial protein per gram wet weight of cells.

Preparation of Mitochondria (Braun shaker).

Cells grown in the fermenter to early stationary phase on 0.5% (v/v) ethanol were always used for this procedure which is described in Chapter 2. The mitochondria were always gradient purified and were used for assays of the Mg^{2+}-dependent ATPase activity.

Measurement of Oxygen Uptake.

Intact Cells.

Yeast cells were grown in shake flask culture on 0.5% (v/v) ethanol and always harvested at early stationary phase. They were normally washed with distilled water and resuspended in the appropriate buffer. A Rank oxygen electrode (Rank Bros., Bottisham, Cambridge) thermostatted at 30°C was used for all measurements of oxygen uptake. The electrode medium consisted of (in order)

1.9 ml buffer 50 mM Sodium phthalate, pH5.0
or 50 mM Tris-maleate, pH 6.5
or 50 mM Tris-HCl, pH 7.4
or 50 mM Tris-H_2SO_4, pH 7.4

0.1 ml cell suspension (1-2 mg dry weight of cells)

10 µl absolute ethanol

All stock solutions of inhibitor or uncoupler were made up in ethanol. All additions were made after a linear rate of oxygen uptake had been established. The initial oxygen concentration was estimated from Chance and Williams, (1967). Tris-H_2SO_4 buffers were used in experiments with TET in the absence of KCl.

Isolated Mitochondria.

A Rank oxygen electrode, thermostatted at 30°C was used for all measurements of oxygen uptake. The electrode medium consisted of (in order)

1.9 ml buffer (0.5 M Sorbitol, 1 mM EDTA, 10 mM Potassium phosphate, 10 mM Tris-maleate, 0.2% (w/v) BSA (Fraction 5), pH 6.5)

0.1 ml mitochondrial suspension (1-2 mg protein)

10 µl substrate (All substrates were buffered to pH 6.5 with sodium hydroxide and/or 10 mM Tris-maleate, and were present at a final concentration of about 5 mM, see text).

Aliquots of ADP were added in order to observe State 3 - State 4 transitions as described in the text. All stock solutions of inhibitors or uncouplers were dissolved in methanol. The initial oxygen content of the medium was estimated from Balcavage and Mattoon, (1968). Addition of methanol alone produced no change in the rate of State 4 oxygen uptake.

ATPase Assay.

The mitochondrial Mg^{2+} - dependent ATPase activity was assayed in a reaction mixture containing 5 mM ATP, 2mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5-10.0 or 50 mM Tris-maleate pH 6.0-7.5, in a final volume of 1 ml. In experiments to measure the effect of TET on the ATPase activity the reaction mixture contained 5 mM ATP, 2 mM $MgSO_4$, 50 mM Tris-H_2SO_4, pH 9.5, in final volume of 1 ml. In all cases 100-200 µg of mitochondrial protein was used for each assay and this was preincubated in the reaction mix for 10 min with inhibitor or uncoupler when necessary. Oligomycin, TET or uncouplers were all added from concentrated stock solutions in methanol. The assays were started by addition of the ATP and were run for 5 min. 1 ml of 10% (w/v) trichloroacetic acid was added to stop the reaction. After centrifugation to sediment the protein, 0.5 ml samples of the supernatant were used for the determination of phosphate according to King, (1932). In a control assay, addition of methanol alone produced only a small decrease in ATPase activity. All % inhibition values were calculated relative to this point.

Dry Weight and Protein Estimations.

Dry weights of intact cells were measured by filtration onto Whatman GF/C glass fibre filter paper (2.4 cm diameter) and dried in an oven at 110-120°C to constant weight for 2-3 days. Protein estimations were by the method of Lowry et al, (1951).

Materials.

Snail gut enzyme ("Helicase") was obtained from Industrie Biologique Francais (Gennevilliers, France). Adenine sulphate, bovine serum albumin oligomycin, ATP and ADP were obtained from Sigma (Sigma (London) Chemical Co., Kingston-on-Thames, U.K.)., TTFB was a gift from Dr. R. B. Beechey (Shell Research Centre, Sittingbourne, Kent); "1799" was a gift from Dr. P. G. Heytler (E. I. Du Pont de Nemours and Co. Inc., Wilmington, Delaware, U.S.A.) and CCCP was obtained from Calbiochem (Los Angeles, California, U.S.A.). TET was a gift from Dr. W. N. Aldridge (M.R.C. Toxicology Research Unit, Carshalton, Surrey). All substrates for mitochondrial oxygen electrode work were obtained from Sigma or from B.D.H. (B.D.H. Chemicals Ltd., Poole, Dorset.). DNP was also obtained from B.D.H. and all other chemicals were of Analytical Reagent grade where necessary.

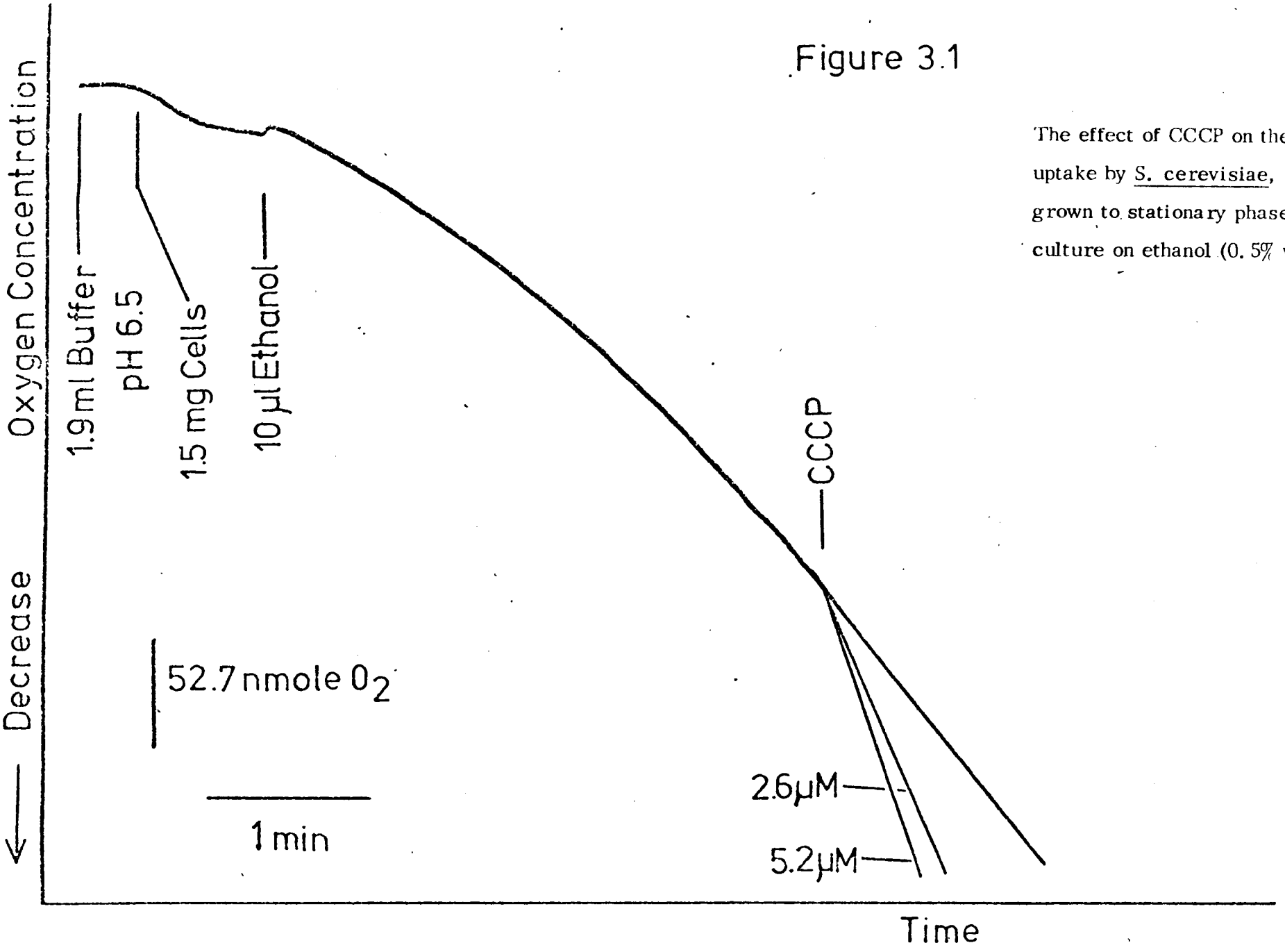
RESULTS.

The Effects of Uncouplers on the Rate of Respiration of Intact Cells of S. cerevisiae.

These experiments were done in an oxygen electrode as described above. Early stationary phase cells were allowed to respire on ethanol and to reach a linear rate before any agent was added. The effect of the addition of CCCP to respiring cells of S. cerevisiae strain D22, at pH 6.5 is shown in Figure 3.1; as expected the rate of oxygen uptake was immediately increased. Linear, uncoupled rates were always obtained upon addition of CCCP or "1799".

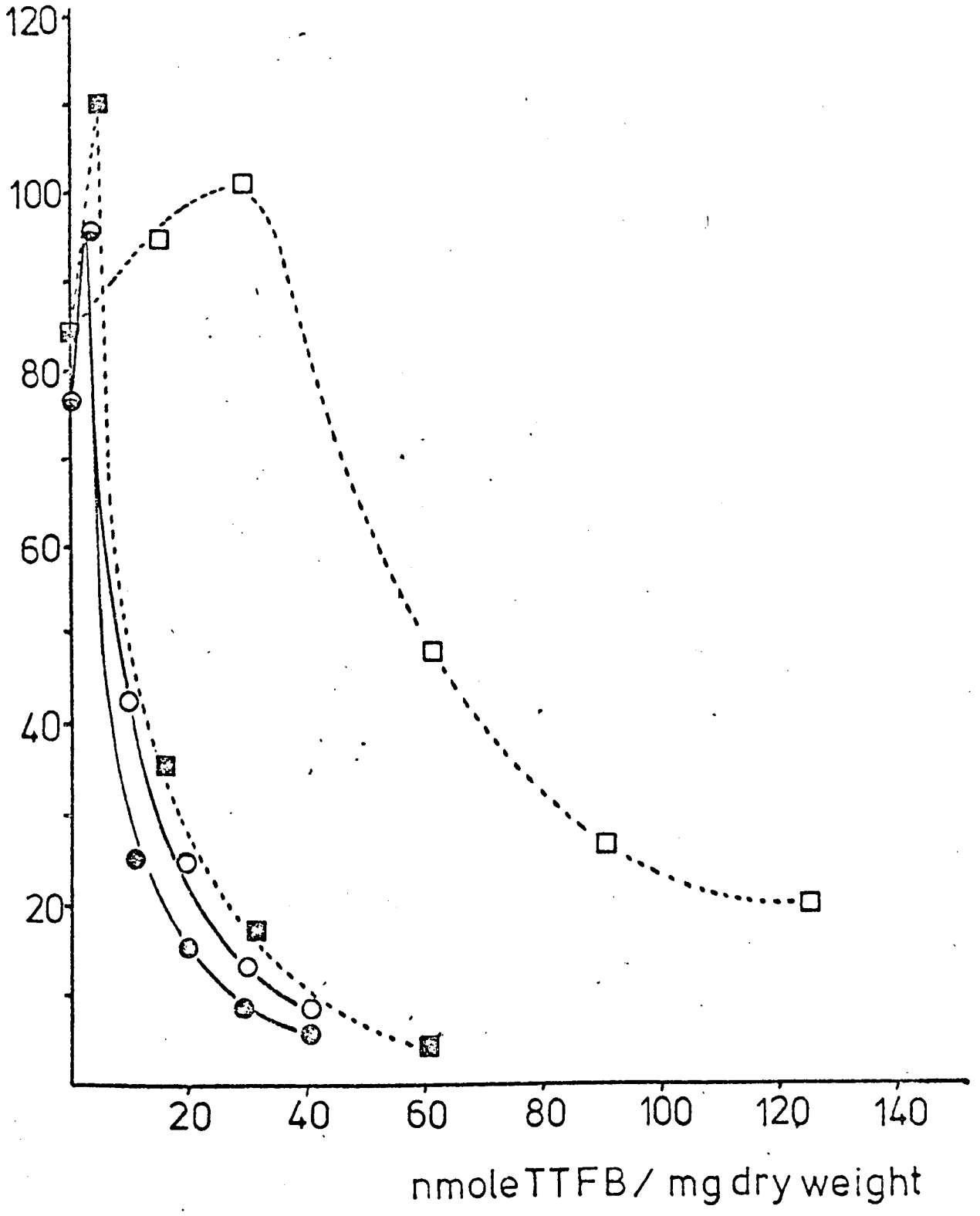
The uncoupler resistant mutants of S. cerevisiae, strain D22, were obtained by plating experiments after ultra-violet irradiation (Griffiths, 1972). Cross resistance determinations, again on plates, showed that the series of cytoplasmic $TTFB^R$ mutants were also resistant to CCCP. However, when grown in liquid culture the effects of TTFB on cell respiration (assayed as in Figure 3.1) are in contrast to those of CCCP or "1799". There was some stimulation of respiration at very low concentraions of TTFB and then, at higher concentrations, increasing inhibition of oxygen uptake. The characteristics of this inhibition had much in common with that produced by TET (Figure 3.7); taking some time to produce a final linear inhibited rate. Figure 3.2 shows the effects of TTFB on the respiration of intact cells of S. cerevisiae, strain D22, and the mutant D22-DCS12 ($TTFB^R$, Class 2). Similar results to those with D22-DCS12 were obtained for all other $TTFB^R$ strains tested ie. D22-DC9, D22-DCS9, D22-CB9 (not shown). The effectiveness of this uncoupler was dependent on the pH of the assay medium and TTFB was more effective in inhibiting respiration at lower pH values (Figure 3.2). However, the resistant strains were always more resistant to TTFB than the wild type at whatever pH was used in the assay (ie.pH 5.0, pH 6.5 or pH 7.4). It is concluded therefore, that TTFB is more effective on intact Saccharomyces yeast in the non-ionised form. From these measurements it is not possible to ascertain directly the site of uncoupler action resulting in inhibition of oxygen uptake. The particular process affected may be localised in either the cell membrane or in the inner mitochondrial membrane and is likely to be concerned with maintainence of the ATP level in the cell and/or with ion

Figure 3.1



The effect of CCCP on the rate of oxygen uptake by S. cerevisiae, strain D22, cells grown to stationary phase in shake flask culture on ethanol (0.5% v/v).

Figure 3.2



The effect of TTFB on the rate of oxygen uptake by S. cerevisiae. The cells were grown to stationary phase on 0.5% (v/v) ethanol in shake flask culture. Assays were done at different pH values.

● - ● D22, pH 5.0 ■ - ■ D22-DCS12, pH 5.0

O - O D22, pH 6.5 □ - □ D22-DCS12, pH 6.5

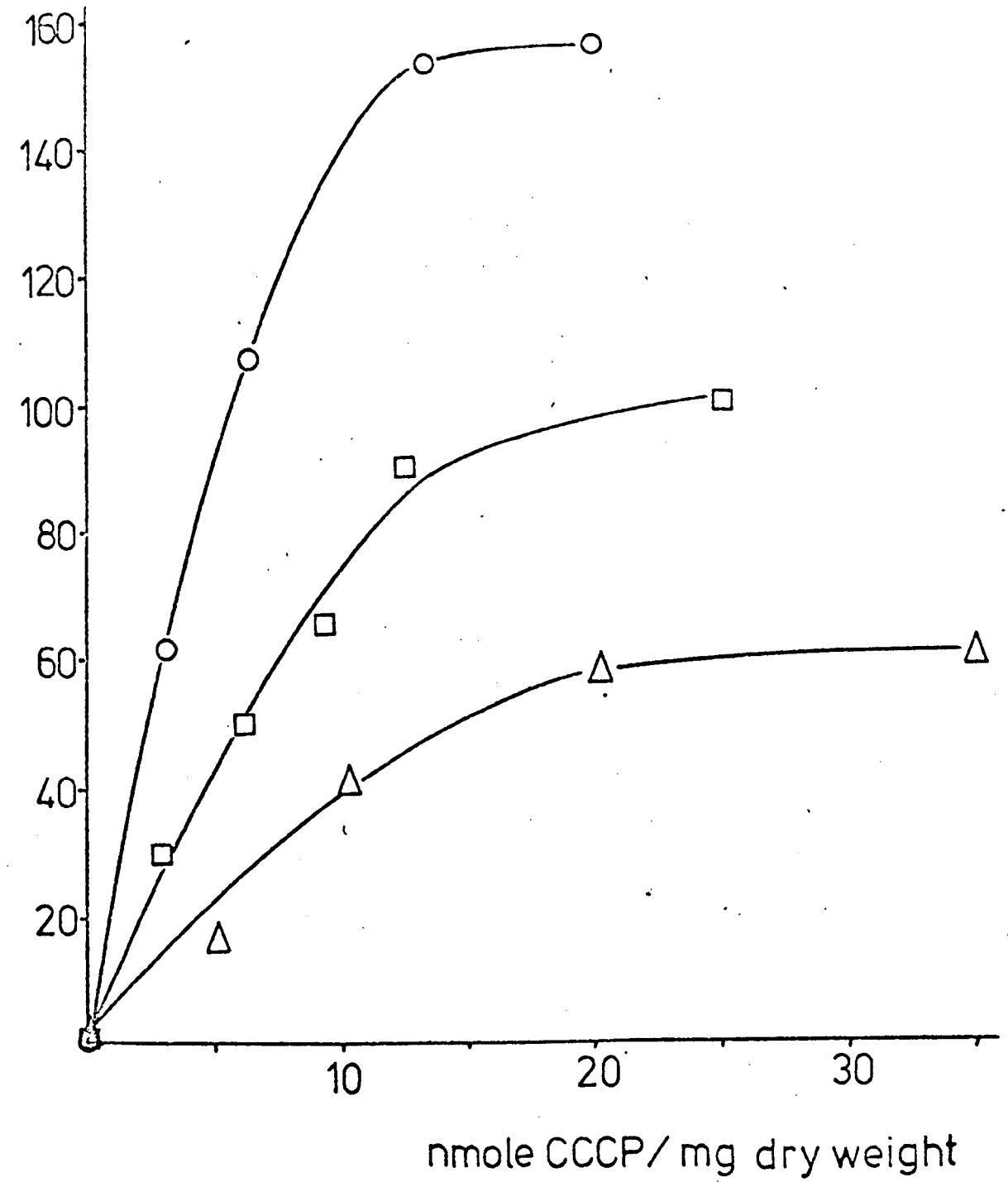
or substrate transport phenomena. However, these observations do show that resistance to TTFB is constitutive, at least in the case of the $TTFB^R$ mutants.

The effects of CCCP on the D22, wild type and selected $TTFB^R$ mutants have been examined. This was done partly to complement the previous cross-resistance studies utilising plating techniques (Griffiths, 1972). These mutants also show resistance to stimulation of oxygen uptake by an uncoupler at the whole cell level. Assays were done as in Figure 3.1 and at pH 5.0, 6.5 or 7.4. The uncoupler was relatively ineffective at stimulating oxygen uptake at pH 7.4. Resistant mutants displayed resistance at all pH values and the results at pH 6.5 for the wild type and the $TTFB^R$ mutants D22-DCS12 and D22-DCS9 are shown in Figure 3.3. The rates of ethanol supported respiration in these early stationary phase cells (Table 3.1) may be compared with the results obtained in Chapter 2 for oxygen uptake during growth on ethanol (Figures 2.4 and 2.5). The respiration of these $TTFB^R$ mutantswas less sensitive to stimulation by CCCP than that of the D22 wild type. This was indicated in two ways; first the maximal % stimulation values for the mutants were less, and second, the mutants were more resistant to the uncoupling activity of lower concentrations of CCCP (Table 3.1). In this assay the Class 2, $TTFB^R$ mutant, D22-DCS12 was more resistant to CCCP than the Class 3 mutant, D22-DCS9.

From cross-resistance assesments using plates, the "1799"^{R} mutants have been characterised as having resistance to CCCP (Table 2.5). The effect of CCCP on the rates of oxygen uptake of intact cells of D22-CB9 and D22-CB19 at pH 6.5 is shown in Figure 3.4. Resistance was not as pronounced as for the $TTFB^R$ mutants and maximal stimulation values approached those of the wild type (Table 3.1). The "1799"^{R}, Class 1 strain, D22-CB9 was more resistant than the Class 2 strain, D22-CB19.

Classification of the various series of drug or uncoupler resistant mutants of S. cerevisiae, strain D22, has been partly on the basis of their cross-resistance to "1799" on plates (Tables 2.2-2.5). It was therefore useful to examine the characteristics of stimulation of respiration by "1799". Assays were performed similar to that shown for the effect of CCCP in Figure 3.1, and at pH 5.0, 6.5 and 7.4. At any pH, "1799" was not such an effective uncoupler as CCCP, but like CCCP its effectiveness in stimulating respiration increased at lower pH values. The effects of "1799" at pH 5.0 on the D22, wild type and selected $TTFB^R$ strains are illustrated in Figure 3.5. On the basis of cross-resistance to "1799"

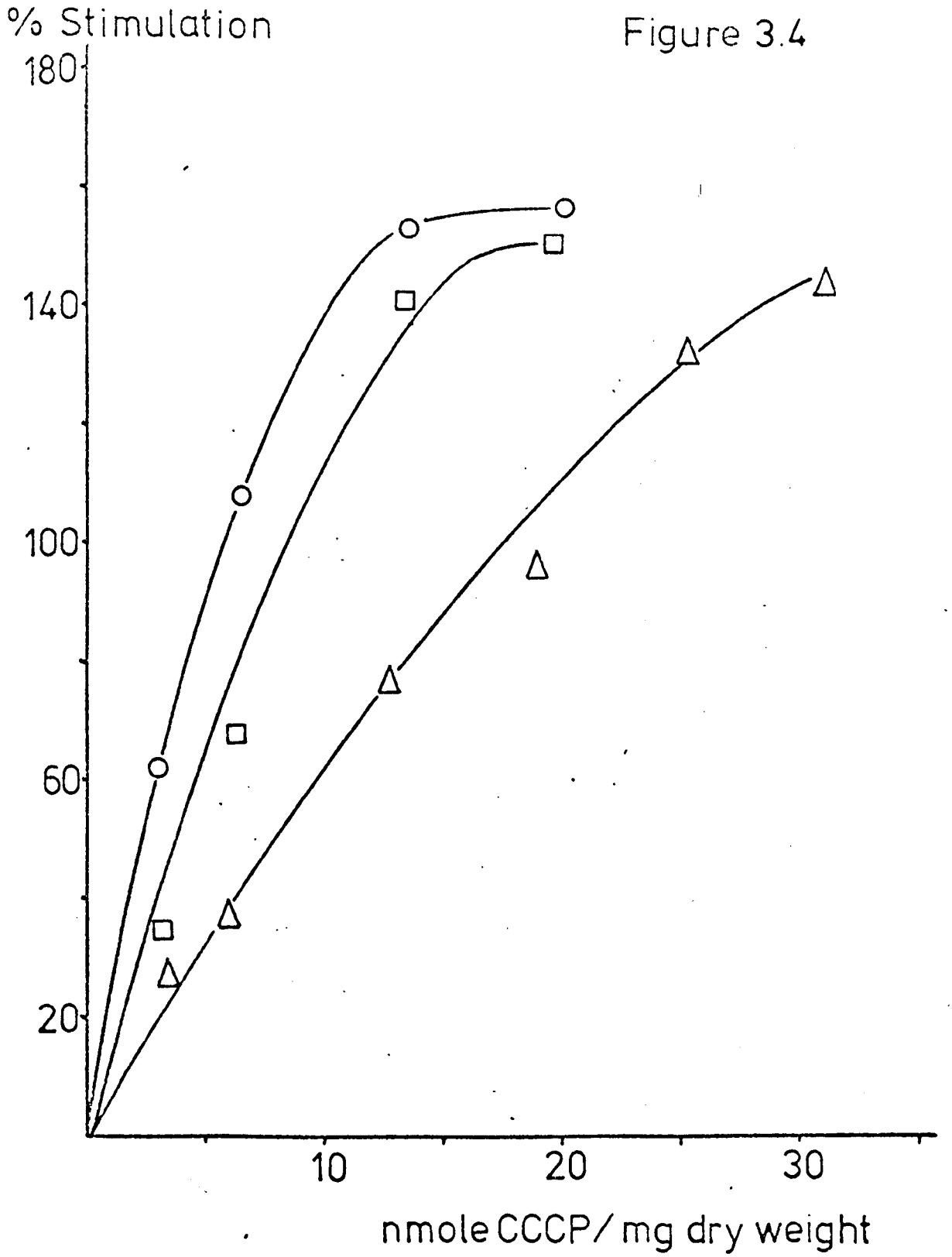
Figure 3.3



Stimulation by CCCP of the rate of oxygen uptake by S. cerevisiae at pH 6.5. The cells were grown to stationary phase on 0.5% (v/v) ethanol in shake flask culture.

O - O D22 □ - □ D22-DCS9 Δ - Δ D22-DCS12.

Figure 3.4



Stimulation by CCCP of the rate of oxygen uptake by S. cerevisiae at pH 6.5.

O - O D22 □ - □ D22-CB19 Δ - Δ D22-CB9

TABLE 3.1

Effect of CCCP at pH 6.5 on the rate of respiration of intact cells of S. cerevisiae.

	Strain	Type	Class	Rate of respiration with no CCCP present.	CCCP added to reach stated % stimulation of respiration			Maximal Values	
					25%	50%	100%	Maximum % stimulation	CCCP concentration at maximum % stimulation
1.	D22	wild type	-	[1]77	[2]1.0	2.5	7.5	155	[2]15.0
2.	D22-DCS12	TTFB^R	2	75	5.5	14.5	N.M.	60	25.0
3.	D22-DCS9	TTFB^R	3	65	2.0	5.0	20.0	100	20.0
4.	D22-CB9	"1799"^{R}	1	65	3.0	8.0	17.5	N.T.	N.T.
5.	D22-CB19	"1799"^{R}	2	80	2.0	4.0	8.5	150	20.0

[1] nmole 0_2/min/mg dry weight. [2] n mole CCCP/mg dry weight.

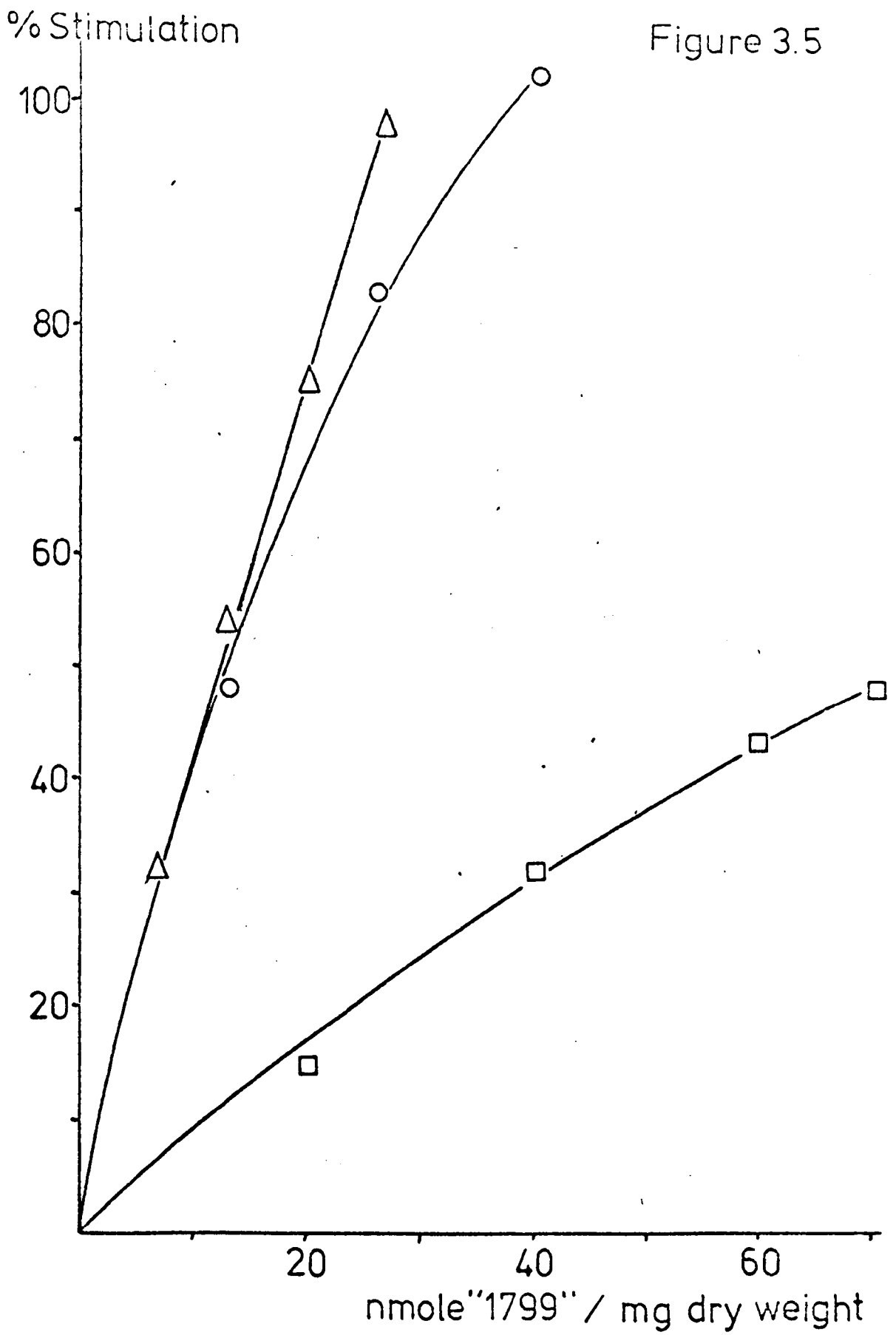
Notes NM = not measurable NT = not tested.

1. All % Stimulation values were calculated relative to rates of respiration with no uncoupler present i.e.

0% Stimulation = 100% activity with no uncoupler present.

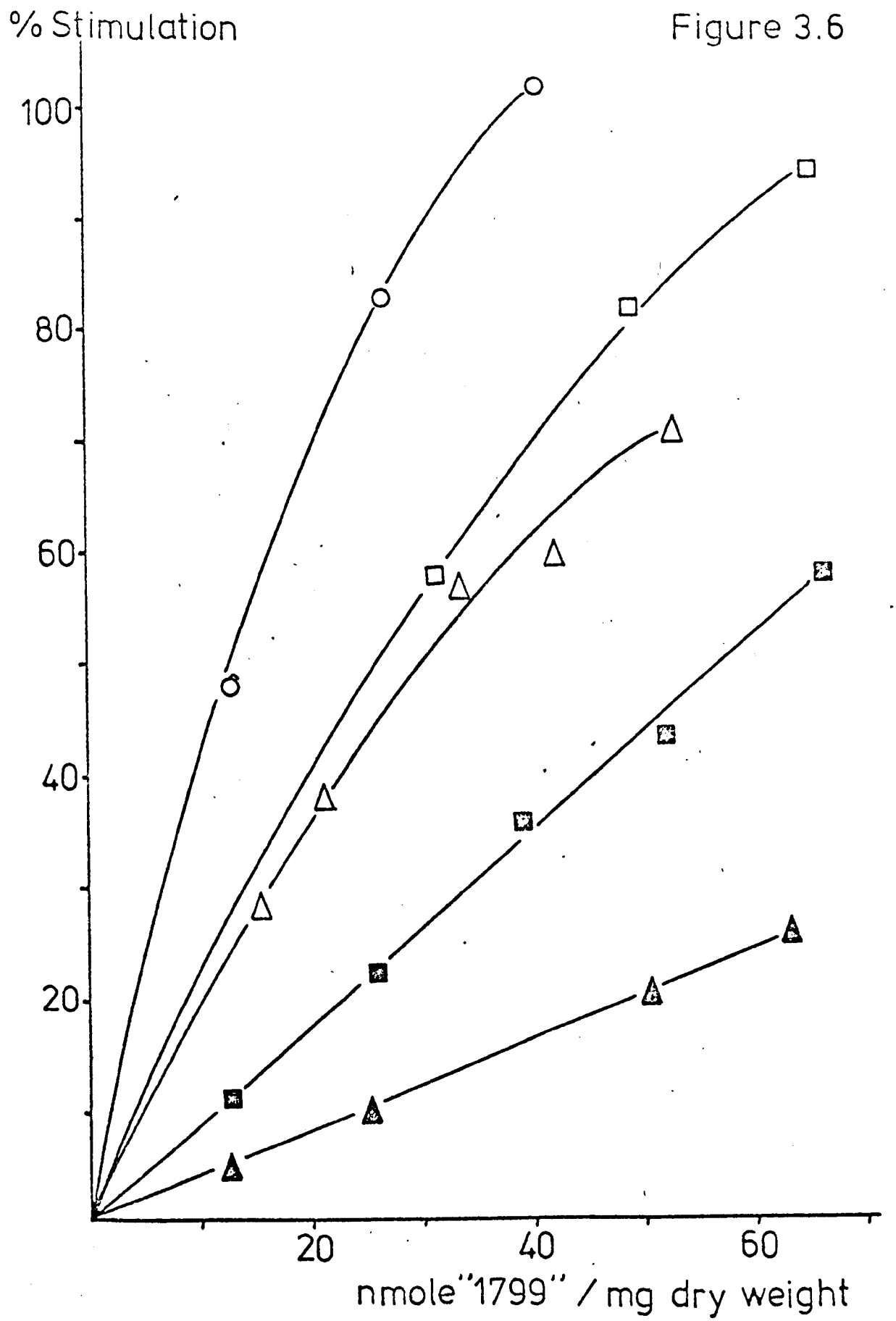
100%Stimultation = 200% activity with no uncoupler present.

Figure 3.5



Stimulation by "1799" of the rate of oxygen uptake by S. cerevisiae at pH 5.0.

O - O D22 □ - □ D22 - DCS12 Δ - Δ D22-DCS9.



Stimulation by "1799" of the rate of oxygen uptake by S. cerevisiae at pH 5.0.

O - O D22. ■ - ■ D22-CB19 ▲ - ▲ D22-CB9

□ - □ D22 - EC1 Δ - Δ D22-EC2

TABLE 3.2

Effect of "1799" at pH 5.0 on the rate of respiration of intact cells of S. cerevisiae.

	Strain	Type	Class	Rate of respiration with no "1799" present	"1799" added to reach stated % stimulation of respiration.		
					25%	50%	100%
1.	D22	wild type	-	[1]77	[2]5.0	12.5	39.0
2.	D22-DCS12	$TTFB^R$	2	75	31.0	75.0	N.T.
3.	D22-DCS9	$TTFB^R$	3	65	5.0	12.5	27.5
4.	D22-CB9	"1799"^{R}	1	65	62.0	N.T.	N.T.
5.	D22-CB19	"1799"^{R}	2	80	29.0	57.0	N.T.
6.	D22-EC1	TET^R	2	70	12.0	26.0	N.T.
7.	D22-EC2	TET^R	3	58	13.0	29.0	N.T.

[1] nmole 0_2/min/mg dry weight [2] nmole "1799"/mg dry weight N.T. = not tested.

Notes

1. % Stimulation values calculated as in Table 3.1.

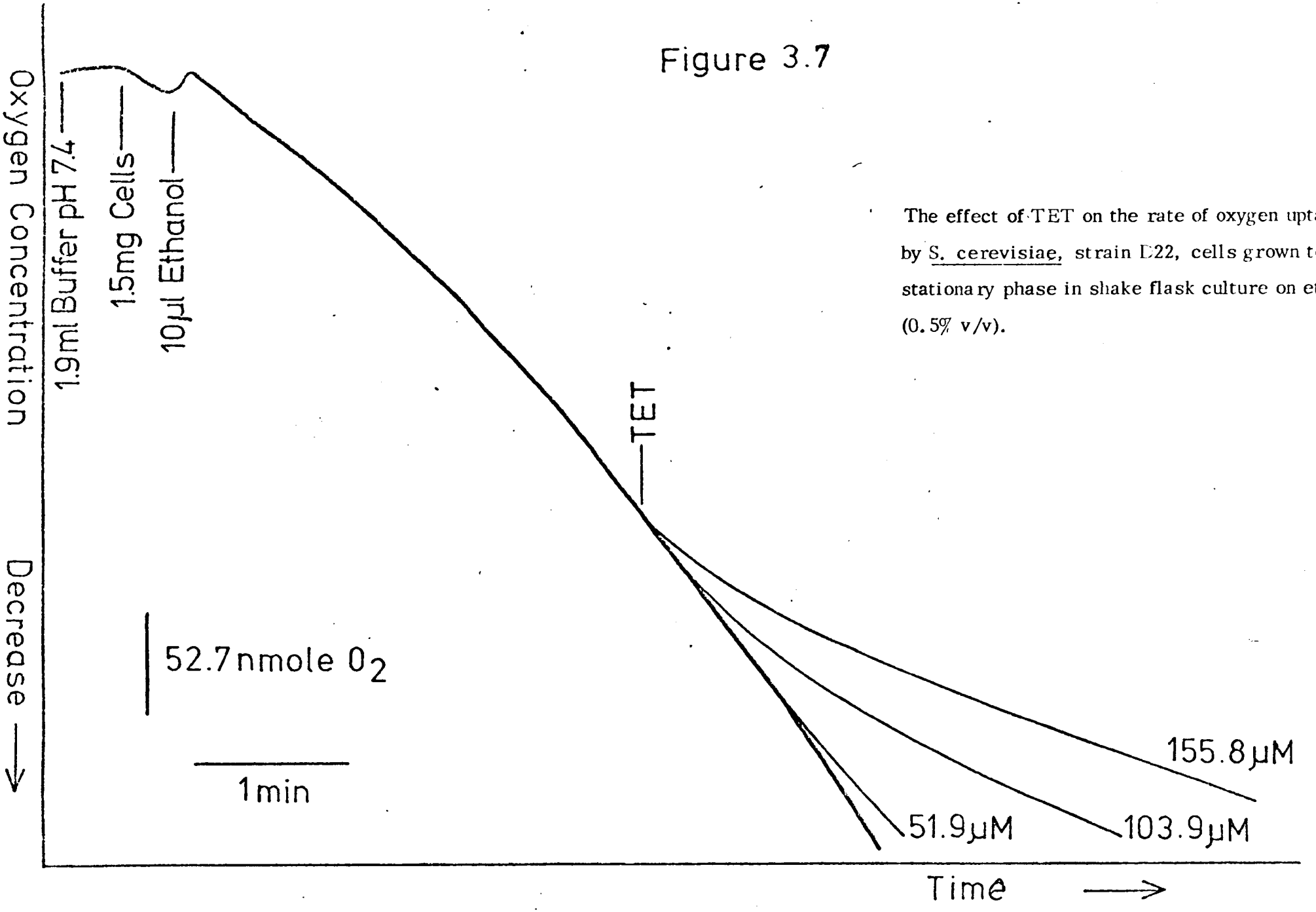
on plates the TTFB^R mutant D22-DCS9 has been classified as a Class 3 (resistant) strain and D22-DCS12 as a (sensitive) Class 2, (Table 2.4). However, in stimulation of respiration the characteristics of D22-DCS9 were very similar to those of the wild type with "1799" while D22-DCS12 showed considerable resistance. No maximum amounts of stimulation were found (Table 3.2).

The series of TET^R mutants that have been isolated from S. cerevisiae, strain D22, (Lancashire and Griffiths, 1971) has also been subdivided on the basis of cross-resistance, on plates, to "1799" (Table 2.3). The effects of "1799" on the cellular respiration of selected "1799"^{R} and TET^R strains are shown in Figure 3.6. Although values of maximal stimulation were again not measureable, all the mutant strains were less sensitive to stimulation of oxygen uptake by "1799" (Table 3.2). Of the "1799"^{R} mutants, D22-CB9 (Class 1) was again the more resistant, in agreement with the results using CCCP (Table 3.1). In all these studies the respiratory rates of all strains tested in the absence of uncoupler were in the same range. The mutation to uncoupler resistance is, on the basis of these studies, constitutive. The primary effect of an uncoupler would be expected to be on the rate of respiration of the mitochondria, these assays show that in most cases the respiratory rate of the mutants is resistant to stimulation.

The Effects of Tri-Ethyl Tin on the Rate of Respiration of Intact Cells of S. cerevisiae.

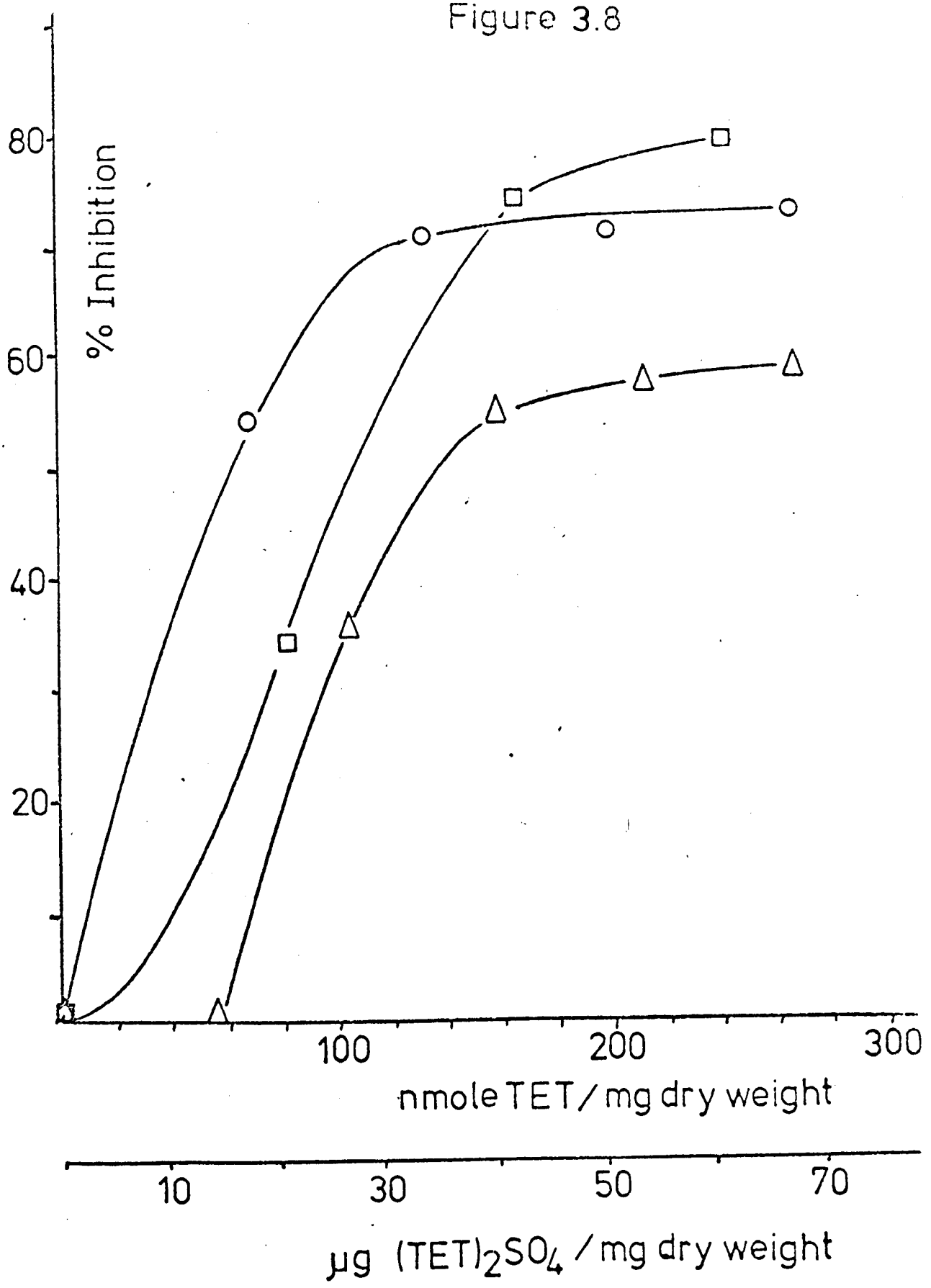
These are complicated by the various properties of this agent. It has an effect on the mitochondrial ATPase similar to that of oligomycin or DCCD and can also catalyse the transport of anions, eg. Cl^-, across the inner mitochondrial membrane (Rose and Aldridge, 1972). Trialkyl tin compounds can also inhibit the transport of adenine nucleotides across the mitochondrial membrane (Harris et al, 1973). The effects of TET on cell respiration have been measured at pH 5.0, 6.5 and 7.4 according to the method shown in Figure 3.7. (Little or no effect was observed at pH 5.0 and the results at pH 6.5 were essentially the same as those at pH 7.4, except that higher concentrations of TET were necessary for comparable inhibition values). The effects of TET on both the D22, wild type and TET^R strains in the presence and absence of 5 mM KCl are shown in Figure 3.8 and 3.9 respectively. The maximum % inhibition for each strain was little affected by KCl but it was noticeable that D22-ECl

Figure 3.7



The effect of TET on the rate of oxygen uptake by S. cerevisiae, strain D22, cells grown to stationary phase in shake flask culture on ethanol (0.5% v/v).

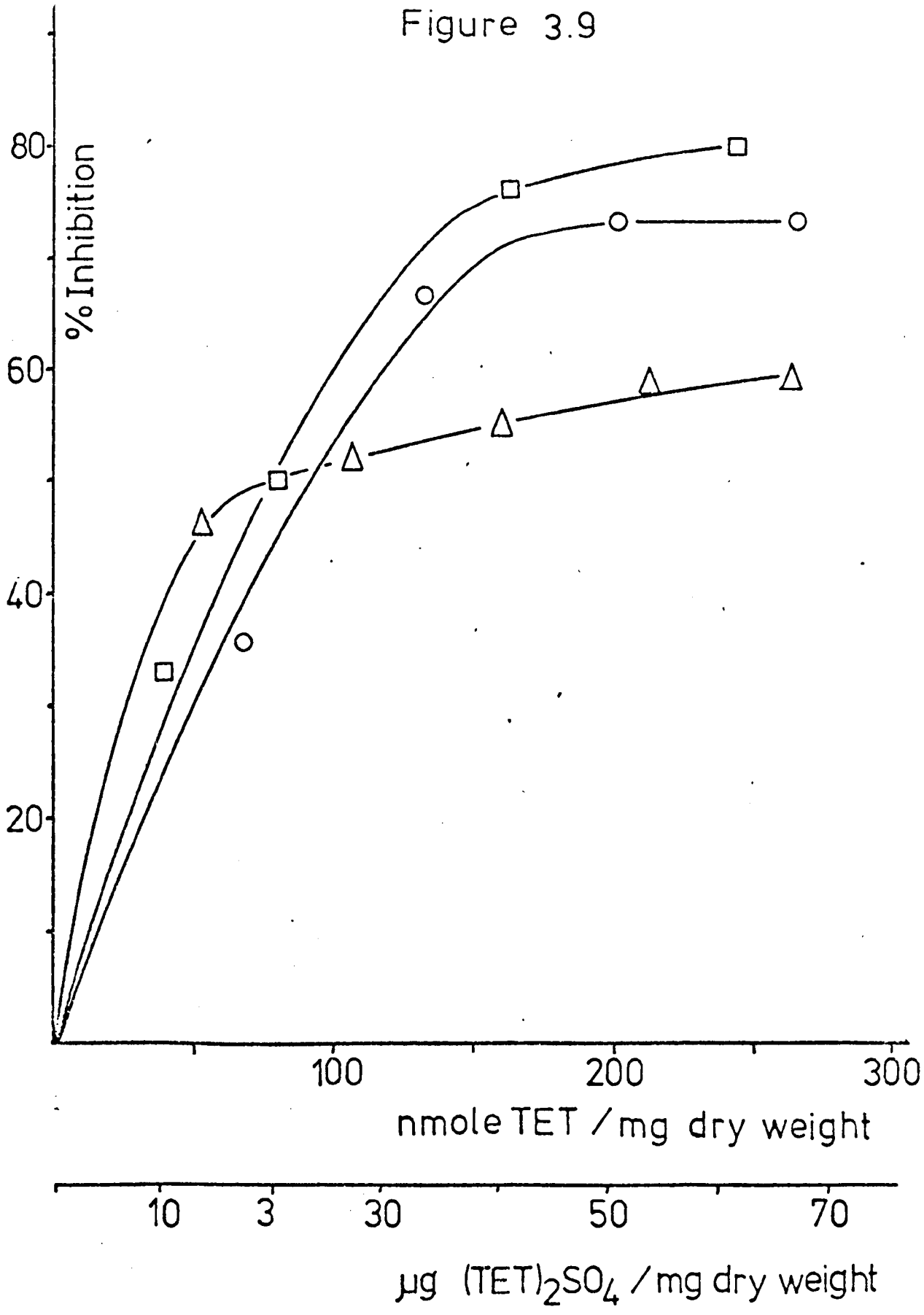
Figure 3.8



Inhibition by TET of the rate of oxygen uptake by S. cerevisiae at pH 7.4 in the presence of 5mM KCl.

O - O D22. □ - □ D22-EC1 Δ - Δ D22 - EC2.

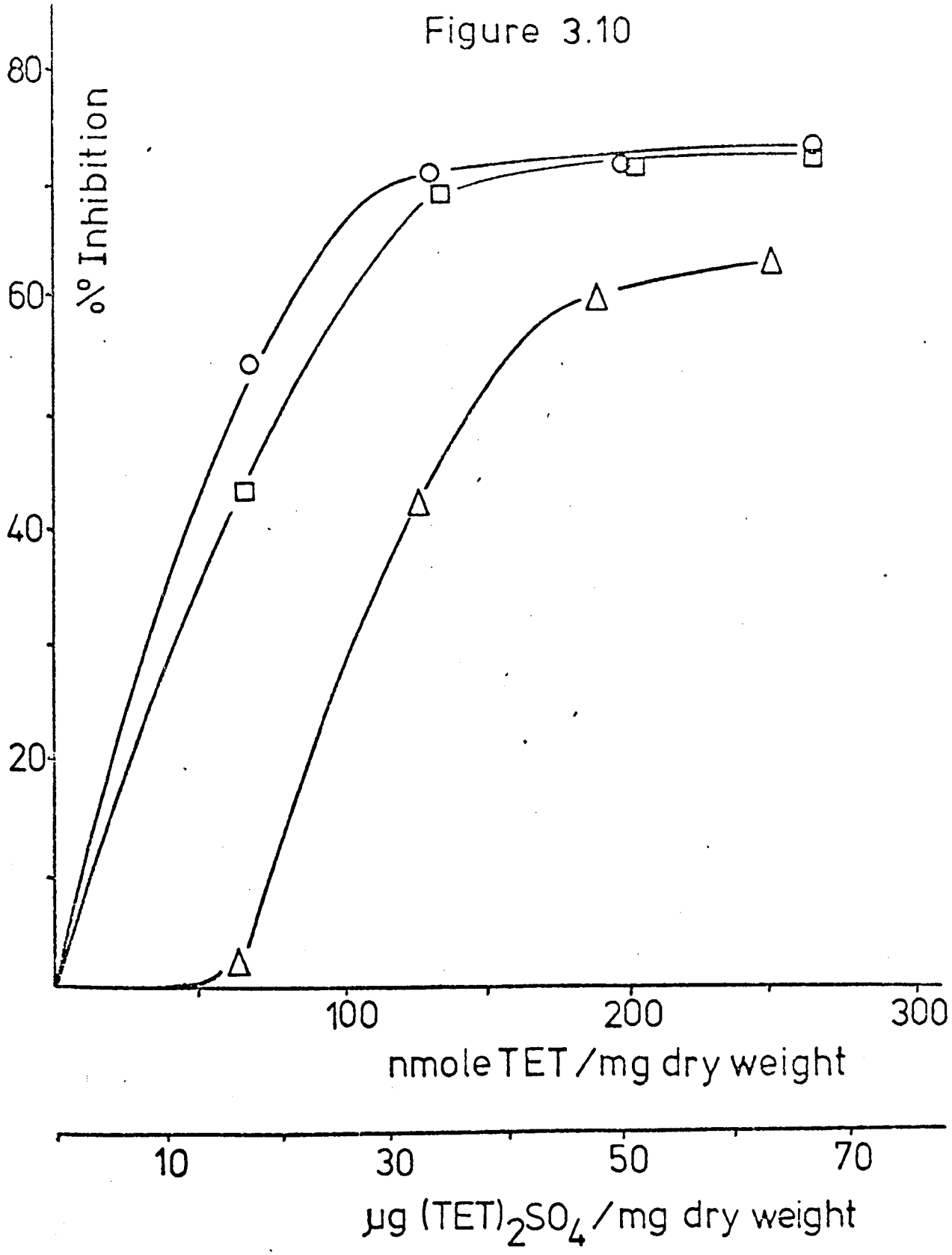
Figure 3.9



Inhibition by TET of the rate of oxygen uptake by S. cerevisiae at pH 7.4.

O - O D22. □ - □ D22 - EC1. Δ - Δ D22 - EC2.

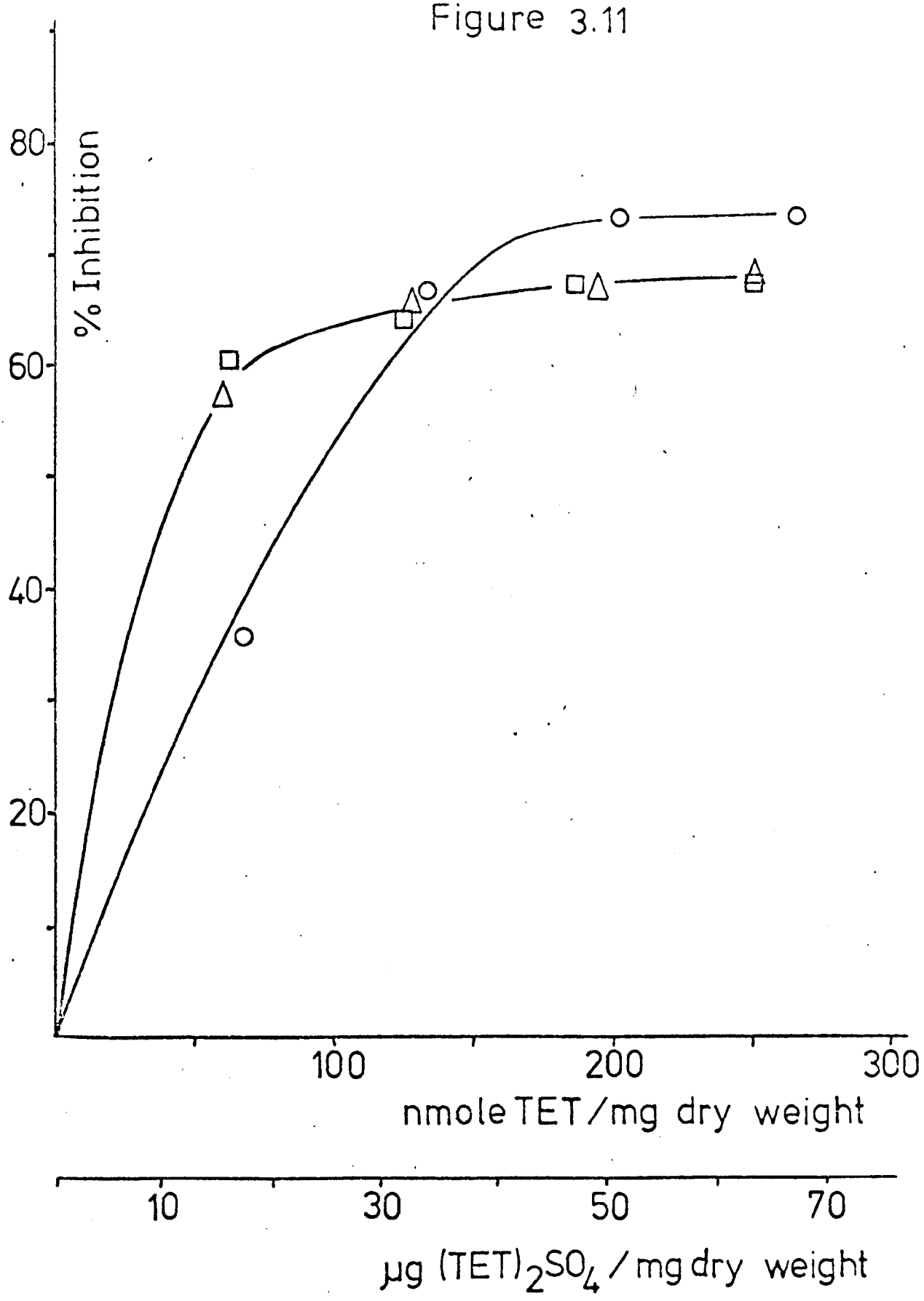
Figure 3.10



Inhibition by TET of the rate of oxygen uptake by S. cerevisiae at pH 7.4 in the presence of 5mM KCl.

O - O D22. □ - □ D22 - CB19. Δ - Δ D22-CB9.

Figure 3.11



Inhibition by TET of the rate of oxygen uptake by S. cerevisiae at pH 7.4.

O - O D22. □ - □ D22-CB19. Δ - Δ D22-CB9.

TABLE 3.3

Effect of TET at pH 7.4 on the rate of respiration of intact cells of S. cerevisiae.

					+ 5mM KCl			No KCl		
	Strain	Type	Class	Rate of respiration ± KCl with no TET present	TET Concentration at 50% Inhibition	Maximum % Inhibition	TET Concentration at maximum % Inhibition	TET Concentration at 50% Inhibition	Maximum % Inhibition	TET Concentration at maximum % Inhibition
1.	D22	wild type	-	[1]77	[2]60	73	[2]260	[2]90	73	[2]200
2.	D22-CB9	"1799"^{R}	1	70	145	63	250	50	68	240
3.	D22-CB19	"1799"^{R}	2	72	80	72	200	50	68	240
4.	D22-EC1	TET^R	2	107	105	80	240	80	80	240
5.	D22-EC2	TET^R	3	61	140	59	260	80	59	260

[1] n moles 0_2/min/mg dry weight [2] n mole TET/mg dry weight.

Notes

1. All % Inhibition values were calculated relative to rates of respiration with no TET present ie.

   0% Inhibition = 100% activity with no TET present.

   100% Inhibition = 0% activity with no TET present.

2. Concentrations are expressed in terms of TET not $(TET)_2SO_4$.

respiration could be inhibited more than the wild type in either case. However, in the absence of KCl, the TET^R mutants D22-EC1 and D22-EC2 were more sensitive to low concentrations of TET than the wild type (Table 3.3). The "1799"^{R} strains D22-CB9 and D22-CB19 were also assayed for resistance of oxygen uptake to inhibition by TET in the presence and absence of 5 mM KCl in order to provide a correlation with plating experiments. These results are shown in Figures 3.10 and 3.11 respectively and follow the same pattern as for the TET^R mutants with respect to the effect of KCl (Table 3.3). It is concluded that resistance to TET may only be expressed in the presence of Cl^- ions. In the presence of KCl low concentrations of TET are without any effect on the respiration of the TET^R strain D22-EC2 and the "1799"^{R} strain, D22-CB9. It is, however, possible that the inhibition curve is shifted to higher concentrations of TET for the mutant strains such that they are much less sensitive than the wild type at lower TET concentrations (Table 3.3).

Oxidative Phosphorylation in Isolated S. cerevisiae Mitochondria.

The mitochondria were prepared by the snail enzyme procedure and were relatively "intact" since there was respiratory control by ADP (State 3 - State 4 transition). Figures 3.12 - 3.14 contain oxygen electrode traces obtained with isolated S. cerevisiae, strain D22, mitochondria. These compare with previous studies on Saccharomyces mitochondria (Ohnishi et al, 1966a, 1967). The best respiratory control and ADP: O ratios were supported by oxoglutarate owing to the existence of the substrate level phosphorylation step. The characteristics of the NAD-linked substrates, ethanol, citrate and pyruvate + malate were similar, with relatively low respiratory control and ADP: O ratios (Table 3.4). These values were similar to those given by succinate supported respiration, but the overall rate of oxygen uptake in this case was much faster (Table 3.4). These mitochondria were prepared from log phase cells and lack functional Site 1 phosphorylation (Ohnishi et al, 1966a) in contrast to those isolated from stationary phase cells (Mackler and Haynes, 1973). Glutamate was not a good substrate, and, as expected, lactate had extremely low respiratory control and ADP: O ratios; since this substrate is oxidised partly through the terminal coupling site only. Compared with other preparations of mitochondria from log phase Saccharomyces sp. cells (Ohnishi et al 1966a, 1967) the mitochondria prepared here consistently had low ADP: O ratios for all substrates. This may be characteristic of the strain or due to damage during the isolation procedure; although the

FIGURES 3.12 - 3.14.

Respiratory control in S. cerevisiae, strain D22, mitochondria. The cells were grown in the fermenter on 1% (v/v) ethanol and harvested in log phase. Mitochondria were prepared by the snail enzyme procedure. Respiration may be supported by various substrates:-

FIGURE 3.12 Ethanol; α-oxoglutarate.

FIGURE 3.13 Citrate; pyruvate-malate.

FIGURE 3.14 Succinate.

Figure 3.12

Oxygen Concentration
Decrease
Time
1.9 ml Buffer
0.425mg mito
4.15 mM Ethanol
125 nmole ADP
41.8 nmole O_2
1 min
1.9ml
Buffer
0.85mg
Mito
5mM
α-OG
ADP
200 nmole
125 nmole ADP
ADP 200 nmole

Figure 3.13

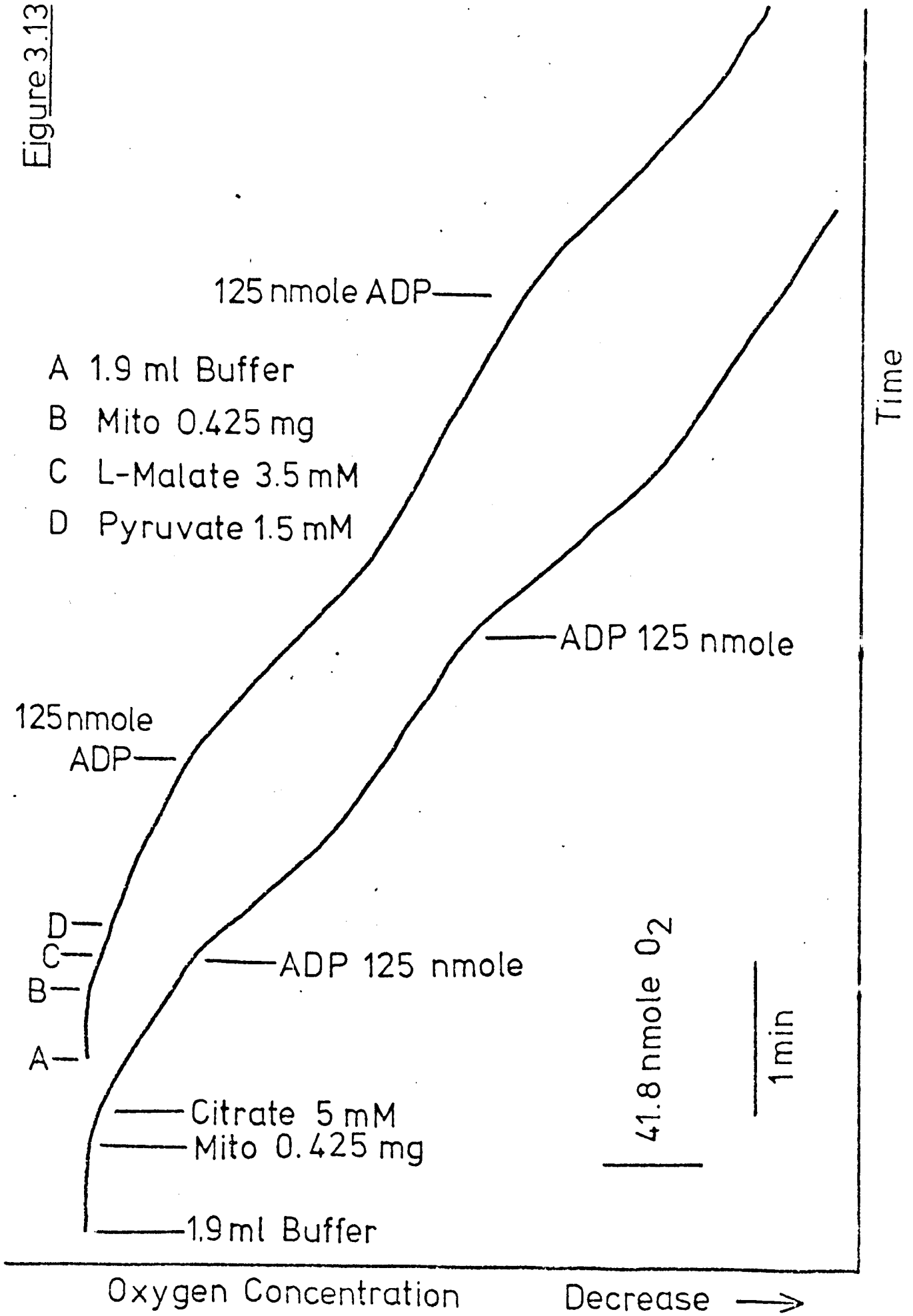

125 nmole ADP
A 1.9 ml Buffer
B Mito 0.425 mg
C L-Malate 3.5 mM
D Pyruvate 1.5 mM
ADP 125 nmole
125 nmole ADP
D
C
B
A
ADP 125 nmole
Citrate 5 mM
Mito 0.425 mg
1.9 ml Buffer
41.8 nmole O_2
1 min
Time
Oxygen Concentration
Decrease

Figure 3.14

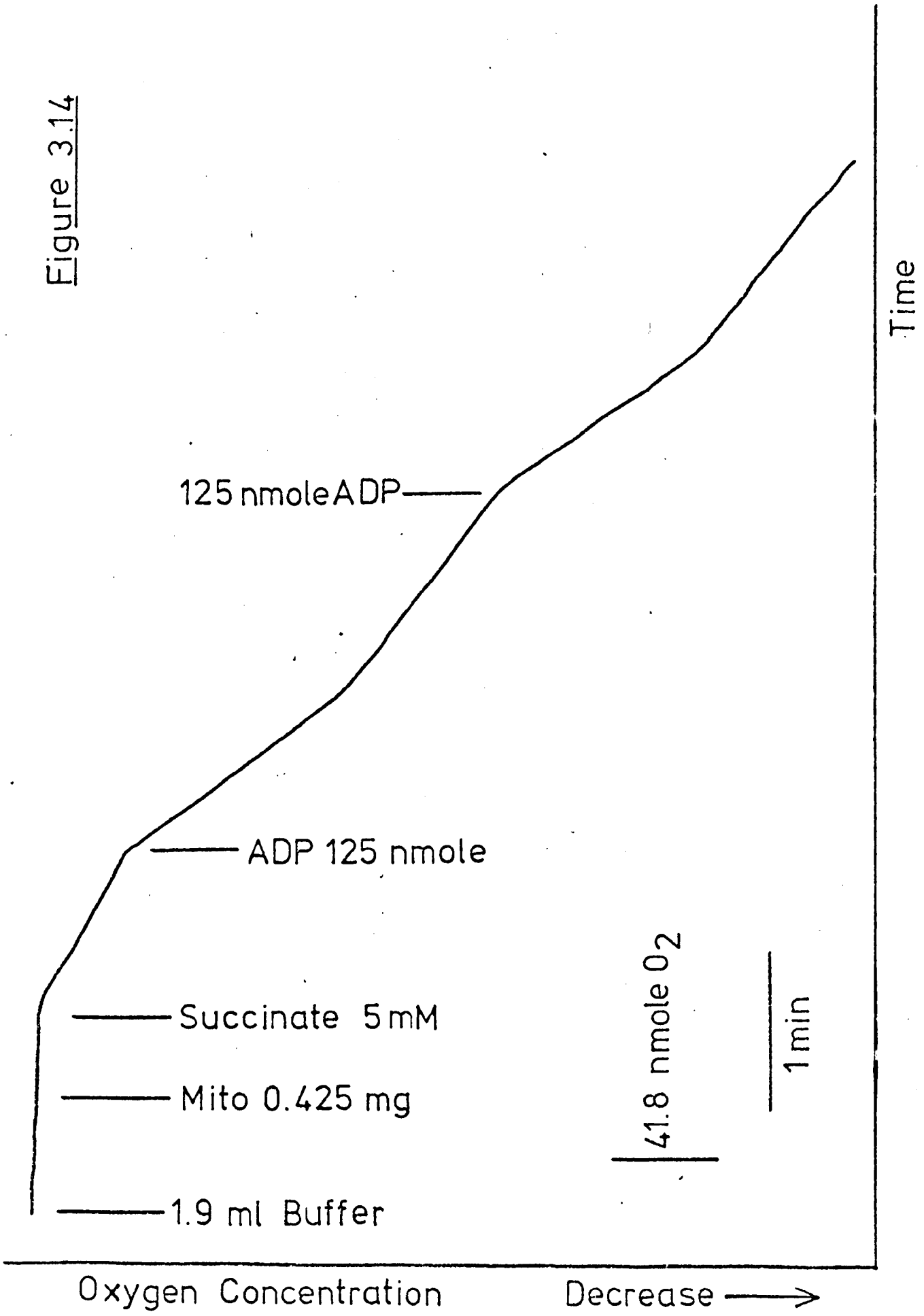

Time
125 nmole ADP
ADP 125 nmole
Succinate 5 mM
Mito 0.425 mg
1.9 ml Buffer
41.8 nmole O_2
1 min
Oxygen Concentration
Decrease

TABLE 3.4.

Oxidative phosphorylation in isolated mitochondria from S. cerevisiae.

Strain	Type	Class	Substrate	State 3 Respiratory Rate	State 4 Respiratory Rate	Respiratory Control	ADP/O	mg protein per assay
D22	wild type	-	[1]OG	[3]135.4	[3]62.7	2.2	1.4	0.85
			Ethanol	142.5	83.7	1.7	0.9	0.425
			Citrate	164.6	98.3	1.7	0.9	0.425
			[2]Pyr-Mal	132.5	81.2	1.6	0.8	0.425
			Glutamate	78.7	68.7	1.1	0.6	0.425
			Succinate	202.0	113.3	1.8	0.7	0.425
			Lactate	118.0	98.3	1.2	0.6	0.425
D22-DCS12	$TTFB^R$	2	OG	106.6	44.4	2.4	1.2	1.05
			Ethanol	106.8	57.0	1.9	0.9	1.05
			Citrate	105.3	56.7	1.9	0.9	1.05
			Pyr-Mal	88.3	53.7	1.6	0.9	1.05
			Glutamate	48.3	41.7	1.2	0.8	1.05
			Succinate	170.0	112.5	1.5	0.7	0.5
			Lactate	107.5	97.5	1.1	0.6	0.5
D22-DC9	$TTFB^R$	2	OG	52.5	30.0	1.8	1.0	1.133
			Ethanol	47.8	34.2	1.4	0.8	1.133
			Citrate	46.2	32.3	1.4	0.8	2.266
			Pyr-Mal	32.3	25.8	1.3	0.6	2.266
			Glutamate	23.7	23.7	1.0	-	2.266
			Succinate	95.8	90.3	1.1	0.5	1.133
			Lactate	42.4	42.4	1.0	-	1.133

Strain	Type	Class	Substrate	State 3 Respiratory Rate	State 4 Respiratory Rate	Respiratory Control	ADP/O	mg protein per assay.
D22-	TTFB^R	3	OG	33.3	20.9	1.6	1.05	2.0
DCS9			Ethanol	30.8	20.9	1.5	0.8	2.0
			Citrate	29.8	22.5	1.3	0.8	2.0
			Pyr-Mal	24.0	16.7	1.4	0.6	2.0
			Glutamate	23.0	23.0	1.0	-	2.0
			Succinate	60.6	50.0	1.2	0.5	1
			Lactate	35.5	35.5	1.0	-	2.0
D22-	"1799"^{R}	1	OG	126.3	54.9	2.3	1.3	1.1
CB9			Ethanol	128.4	75.1	1.7	0.9	1.1
			Citrate	148.4	86.3	1.7	1.0	1.1
			Pyr-Mal	127.8	78.4	1.6	0.8	1.1
			Glutamate	76.2	70.0	1.1	0.6	1.1
			Succinate	172.2	106.1	1.7	0.7	0.55
			Lactate	109.3	94.2	1.2	0.5	1.1
D22-	TET^R	3	OG	65.3	37.1	1.8	1.2	1.9
EC2			Ethanol	53.6	37.2	1.4	0.8	1.9
			Citrate	56.4	37.4	1.5	0.8	1.9
			Pyr-Mal	44.2	34.0	1.3	0.7	1.9
			Succinate	104.3	92.3	1.1	0.5	0.95

[1] OG = α - oxoglutarate. [2] Pyr-Mal = Pyruvate + Malate. [3] nmole 0_2/min/mg protein

Notes

1. State 3 is rapid respiration and phosphorylation not limited by lack of substrate or phosphate acceptor.

2. State 4 is an aerobic state with low respiration limited by lack of Pi or acceptor but not by lack of substrate.

3. Respiratory control = $\frac{\text{State 3 respiratory rate}}{\text{State 4 respiratory rate}}$

4. Results for D22 are analyses of Figures 3.12 - 3.14.

respiratory control values were in the same range as those quoted by Ohnishi et al, (1966a). It was not possible to obtain mitochondria from stationary phase cells of S. cerevisiae, strain D22, because at this stage of growth in batch culture the cell wall of this yeast was insensitive to digestion by snail gut enzyme.

The characteristics of mitochondria isolated from $TTFB^R$, "1799"^{R} and TET^R mutants of S. cerevisiae, strain D22, are compared with those of the wild type (Table 3.4). In the case of the $TTFB^R$, Class 2, strain D22-DCS12 the rates of oxidation of all substrates, except lactate, were 20-30% less than the corresponding rates in wild type mitochondria. With D22-DC9 and D22-DCS9 respiratory rates were down to 40-50% of the wild type values with all substrates and no respiratory control was observed with either glutamate or lactate as substrate. The properties of the mitochondria from the Class 1, "1799"^{R} mutant, D22-CB9 were comparable with the wild type but the rates of respiration in D22-EC2 (TET^R, Class 3) mitochondria were about 50% of the corresponding wild type rates for all substrates. In no case did a mutation to uncoupler or TET resistance produce an increase in the phosphorylation efficiency of isolated mitochondria. Normally the ADP; O ratios for the mutants were the same as, or slightly less than, the corresponding wild type value.

The Effects of Uncouplers on the Rate of Respiration of Isolated Mitochondria from S. cerevisiae.

The effects of uncouplers on isolated mitchondria were assayed by measurement of the stimulation of the "State 4" rate of oxygen uptake, produced by the addition of uncoupling agent. The mitochondria were first taken through a "State 3 - State 4" transition by addition of ADP, in order to provide a common reference point, and to provide a control to show that the mitochondria were capable of the same amount of uncoupling in each assay (Figure 3.15). Although oxoglutarate as substrate supports the best respiratory control values it could not be used in this assay owing to interference by the substrate level phosphorylation step, and ethanol was used instead. (If oxoglutarate was used the rate of the "State 4" respiration was progressively inhibited by increasing concentration of CCCP). The higher the respiratory control the greater the range of measurement that can be used for stimulation of the "State 4" rate by uncoupler (Margolis et al, 1967) and the more accurate the results. It is also possible to use a phosphate trap to measure the amount of ATP produced by addition of ADP in the presence of uncoupler.

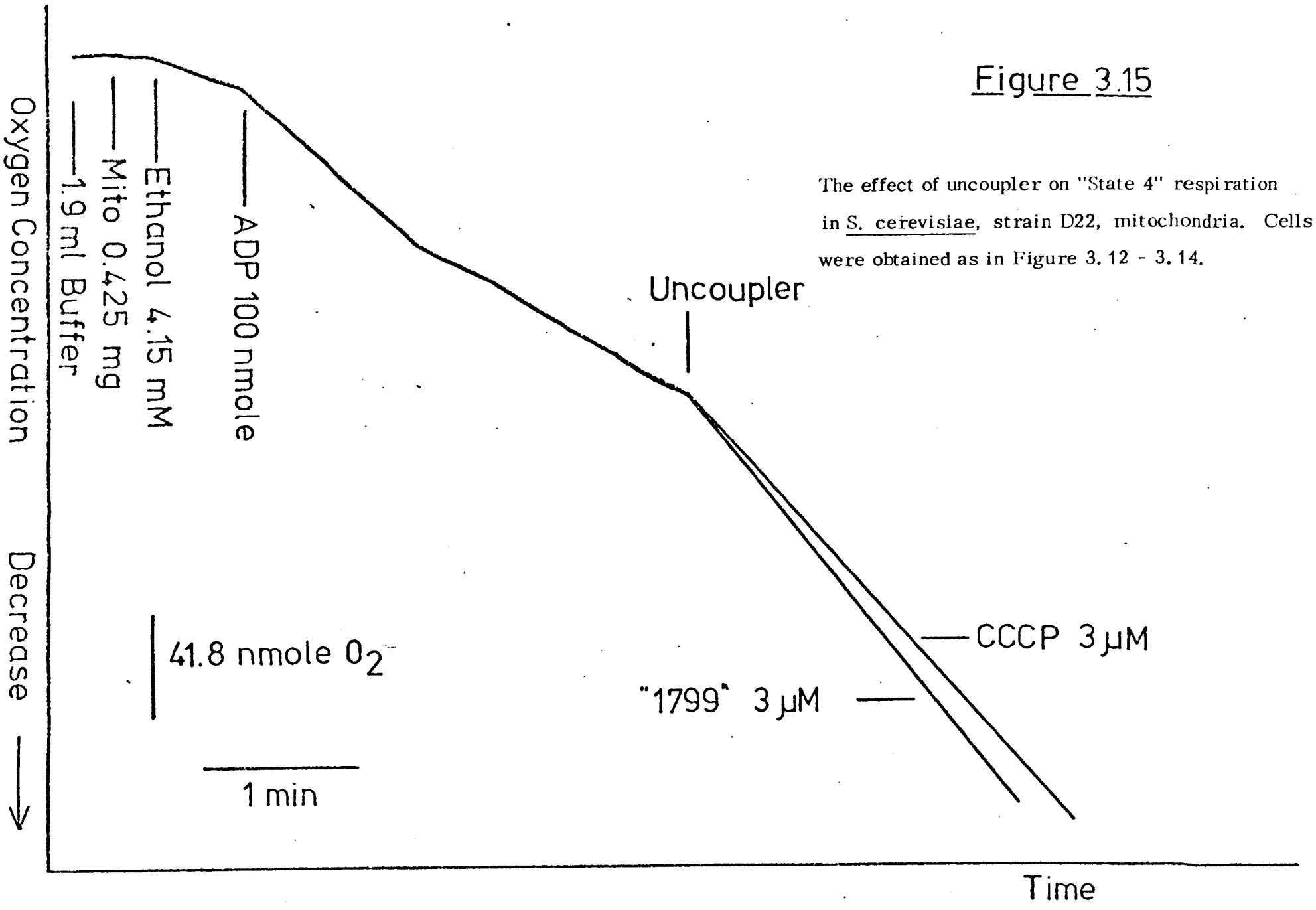


Figure 3.15
The effect of uncoupler on "State 4" respiration in S. cerevisiae, strain D22, mitochondria. Cells were obtained as in Figure 3.12 - 3.14.
Oxygen Concentration
Decrease
1.9 ml Buffer
Mito 0.425 mg
Ethanol 4.15 mM
ADP 100 nmole
Uncoupler
CCCP 3 µM
"1799" 3 µM
41.8 nmole O_2
1 min
Time

FIGURE 3.16

Stimulation of S. cerevisiae mitochondrial "State" 4 respiration by CCCP.

O - O D22.

□ - □ D22-DCS12.

Δ - Δ D22-DCS9.

X - X D22-DC9.

Figure 3.16

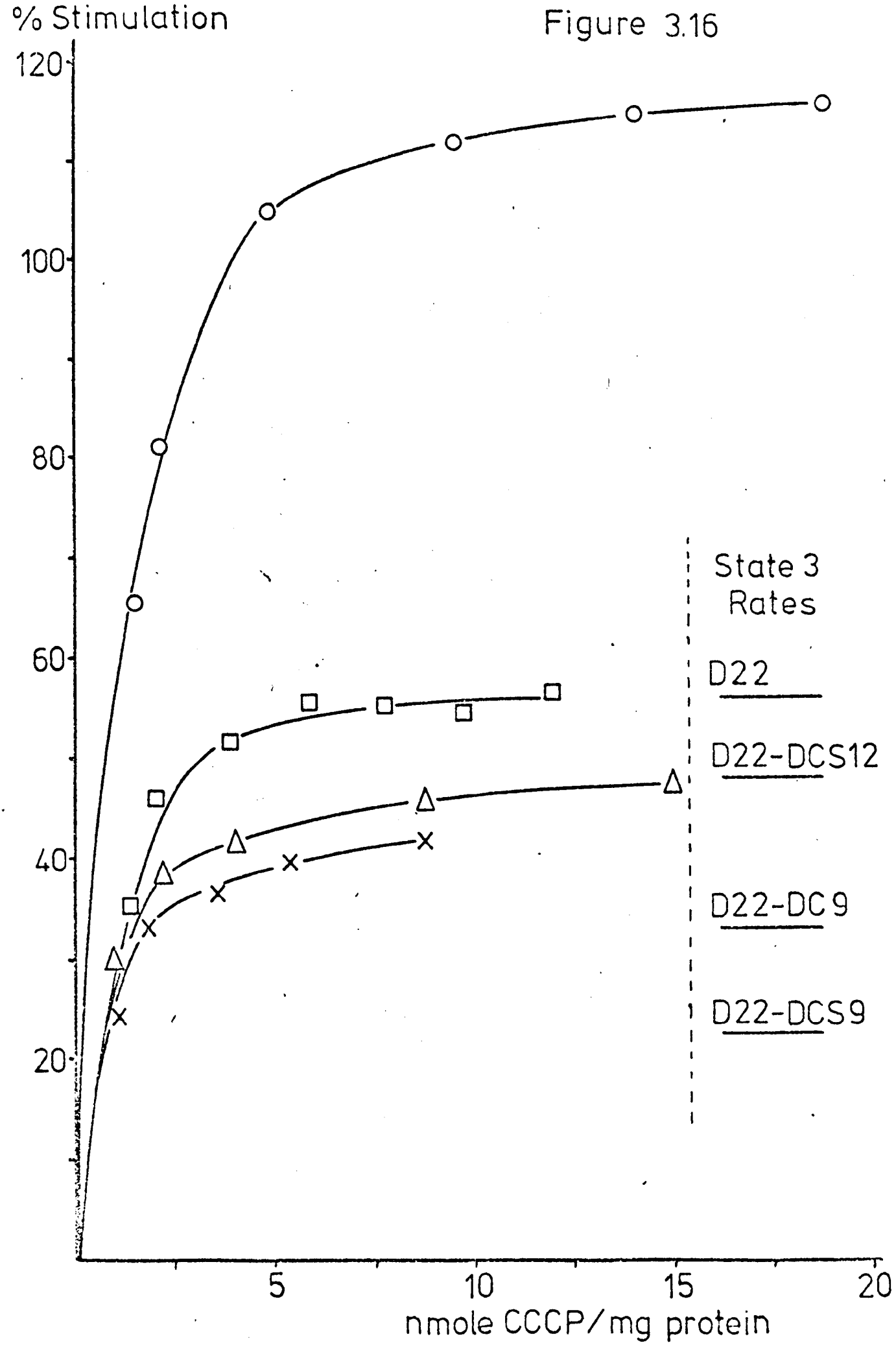

% Stimulation
120
100
80
60
40
20
5
10
15
20
nmole CCCP/mg protein
State 3 Rates
D22
D22-DCS12
D22-DC9
D22-DCS9

TABLE 3.5.

Stimulation of State 4 respiration in S. cerevisiae mitochondria by CCCP.

	Units	D22 wild type	Strain, Type and Class			
			D22-DC9 $TTFB^R$ Class 2	D22-DCS12 $TTFB^R$ Class 2	D22-DCS9 $TTFB^R$ Class 3	D22-CB9 "1799"^{R} Class 1
Mitochondria present in assay	mg protein	0.425	1.133	1.05	2.0	1.1
Average State 3 rate	nmole0_2/min/mg protein	128	44	93	27	129
Average State 4 rate	nmole0_2/min/mg protein	82	33	63	22	77
%Stimulation of State 4 rate to equal State 3 rate		56	33	48	23	68
CCCP concentration to stimulate State 4	nmole CCCP/mg protein	0.9	1.8	2.5	0.6	7.5
rate to equal state 3 rate.	μM CCCP	0.2	1.0	1.3	0.6	4.2
CCCP concentration to stimulate State 4	nmole CCCP/mg protein	0.25	0.5	0.75	0.25	2
rate to equal half State 3 rate.	μM CCCP	0.05	0.3	0.4	0.25	1.1
Maximum % Stimulation of State 4 rate.		115	42	56	46	N.M.
CCCP concentration on attaining	nmole CCCP/mg protein	10	10	10	10	N.M.
maximal stimulation of State 4 rate	μM CCCP	2.1	5.6	5.3	10	N.M.

N.M. = Not Measurable.

Notes.

1. (a) $\%\ \text{Stimulation} = \left(\frac{\text{uncoupled rate}}{\text{State 4 rate}} \times 100\right) - 100$

(b) $\%\ \text{Stimulation to} = \text{State 3 rate} = \left(\frac{\text{State 3 rate}}{\text{State 4 rate}} \times 100\right) - 100$

(c) 0% Stimulation = State 4 rate.

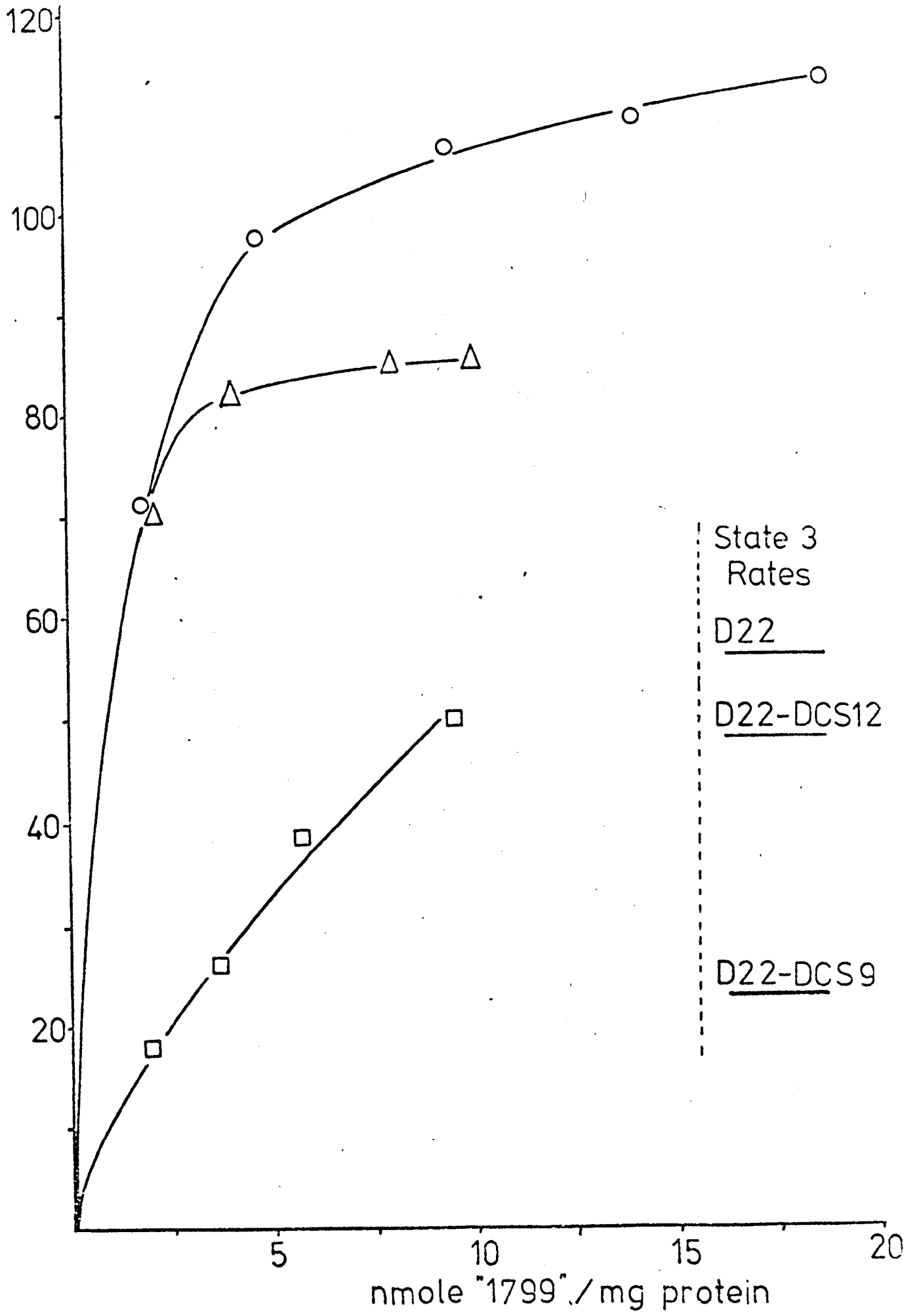
2. These results are obtained from Figures 3.16 and 3.18.

FIGURE 3.17

Stimulation of S. cerevisiae mitochondrial "State" 4 respiration by "1799".

O - O D22. □ - □ D22-DCS12. Δ - Δ D22-DCS9.

Figure 3.17


% Stimulation
120
100
80
60
40
20
5
10
15
20
nmole "1799"./mg protein
State 3 Rates
D22
D22-DCS12
D22-DCS9

The percentage stimulation of the "State 4" rate, for the D22 wild type and selected $TTFB^R$ mutant mitochondria, is titrated against CCCP in Figure 3.16. The "State 4" rate of respiration was maximally stimulated past the "State 3" rate in every case. There was an initial large stimulation of oxygen uptake by CCCP and at higher concentrations the curves tended to plateaux. This maximal amount of stimulation is always less for the mutants than for the wild type (Table 3.5). This is not a reflection of the lower initial rates of respiration of the mutant mitochondria since % stimulation values are quoted and not absolute rates. Mitochondrial respiration in the mutants was also less sensitive to low concentrations of CCCP than that of the wild type (Table 3.5).

The effects of "1799" on the mitochondrial respiration of $TTFB^R$ mutants (Figure 3.17) are comparable with those on intact cell respiration (Figure 3.5) Marked resistance to stimulation was seen in D22-DCS12 mitochondria (Class 2) and only slight resistance in the case of mitochondria from the Class 3 mutant D22- DCS9. This again is in contrast to the cross-resistance data obtained using plating techniques (Table 2.4). The D22 and D22-DCS9 mitochondria were stimulated to maximal rates of respiration by "1799" but D22-DCS12 did not show a plateau over the range tested (Table 3.6). D22-DCS9 mitochondria were as sensitive to low concentrations of "1799" as those of the wild type strain.

The effects of CCCP or "1799" on "State 4" respiration in mitochondria from D22-CB9 ("1799"^{R} Class 1) are shown in Figure 3.18. In either case the % stimulation did not plateau over the concentration range studied and respiration was, as expected, markedly resistant to stimulation even at low concentrations of "1799", (Table 3.6) and only slightly less resistant to CCCP (Table 3.5). The effect of CCCP on mitochondrial oxygen uptake in the Class 3 TET^R strain, D22-EC2 was not tested. This strain did however show some resistance to stimulation by "1799" in this assay (Figure 3.18, Table 3.6). These data, summarised in Tables 3.5 and 3.6, show that resistance to the effects of uncouplers can occur at the mitochondrial level. These studies generally correlate with data on the action of uncouplers on intact cell respiration (Tables 3.1 and 3.2) although not necessarily with the results obtained from cross-resistance studies using plating techniques (Griffiths, 1972; Tables 2.1 - 2.5). On the basis of these observations "1799" is not such an effective uncoupler as CCCP in S. cerevisiae mitochondria.

FIGURE 3.18

Stimulation of S. cerevisiae mitochondrial "State" 4 respiration by uncouplers.

● - ● D22, CCCP

O - O D22, "1799"

Δ - Δ D22 - EC1, "1799".

■ - ■ D22-CB9, CCCP.

□ - □ D22-CB9, "1799".

Figure 3.18

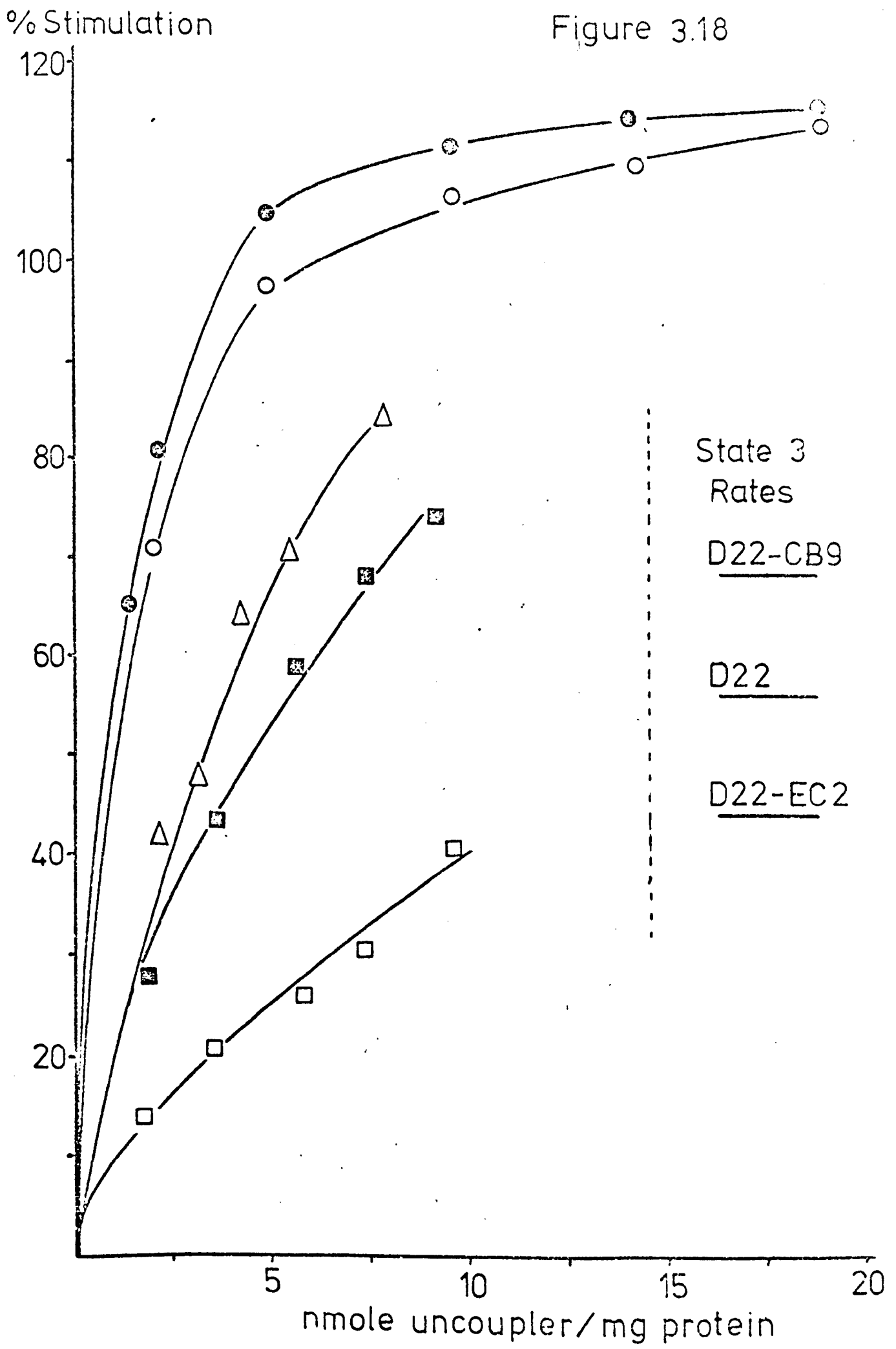

% Stimulation
120
100
80
60
40
20
5
10
15
20
nmole uncoupler/mg protein
State 3 Rates
D22-CB9
D22
D22-EC2

TABLE 3.6.

Stimulation of State 4 respiration in S. cerevisiae mitochondria by "1799".

	Units	Strain, Type and Class				
		D22 wild type	D22-DCS12 $TTFB^R$ Class 2	D22-DCS9 $TTFB^R$ Class 3	D22-CB9 "1799"^{R} Class 1	D22-EC2 TET^R Class 3
Mitochondria present in assay	mg protein	0.425	1.05	2.0	1.1	1.9
Average State 3 rate.	nmole0_2/min/mg protein	128	93	27	129	56
Average State 4 rate.	nmole0_2/min/mg protein	82	63	22	77	39
% Stimulation of State 4 rate to equal State 3		56	48	23	68	44
"1799" concentration to stimulate State 4 rate to equal State 3 rate	nmole"1799"/mg protein μM "1799"	1.0 0.2	8.8 4.6	0.25 0.25	N.M. N.M.	2.5 2.4
"1799" concentration to stimulate State 4 rate to equal half of State 3 rate	nmole "1799"/mg protein μM "1799"	0.5 0.1	3.0 1.6	N.M. N.M.	8.0 5.0	0.75 0.72
Maximum % Stimulation of State 4 rate. "1799" concentration on attaining maximal stimulation of State 4 rate	 nmole "1799"/mg protein μM "1799"	114 18.8 4.0	N.M. N.M. N.M.	85 10.0 10.0	N.M. N.M. N.M.	N.M. N.M. N.M.

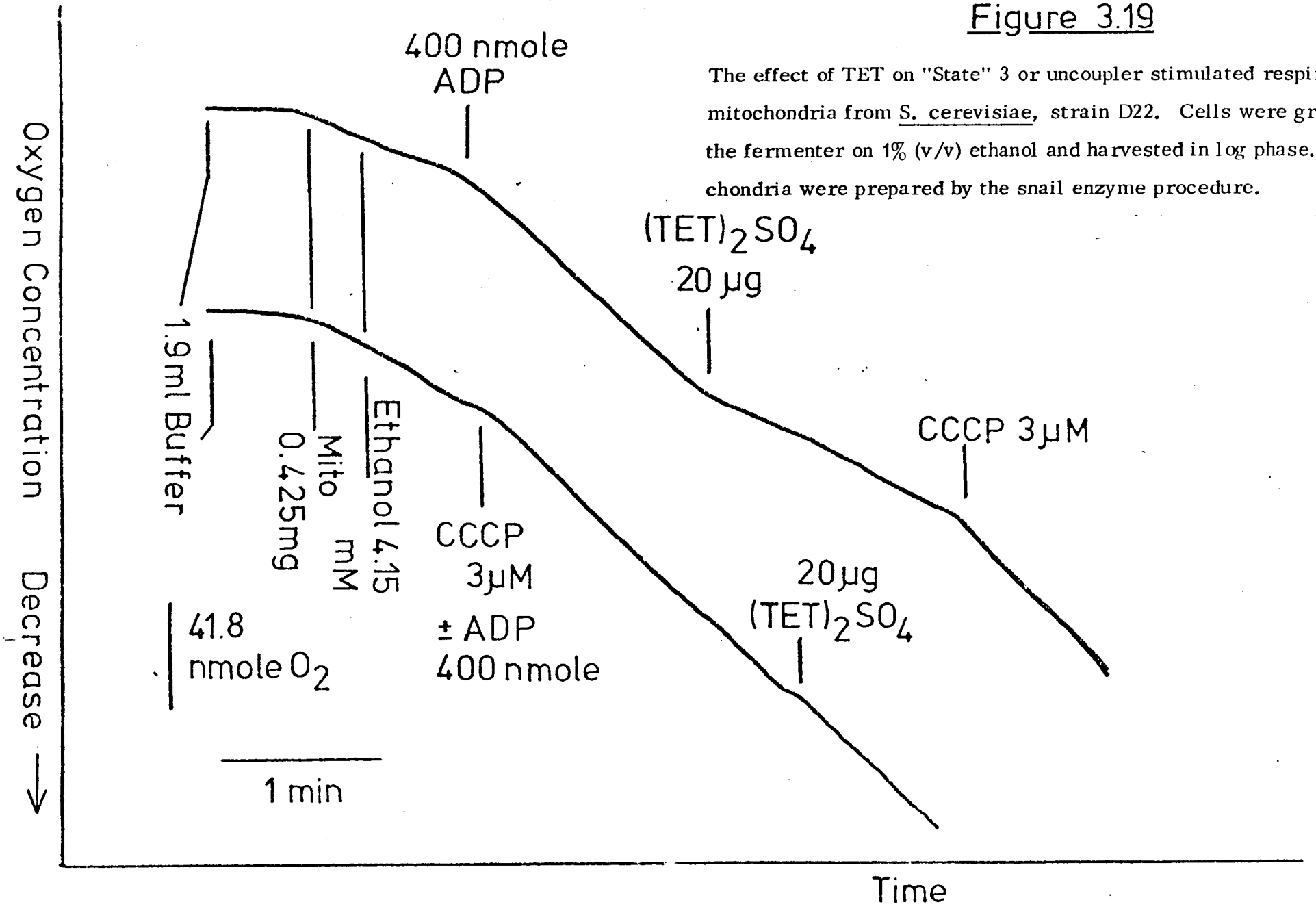
N.M. = Not Measurable.

Notes.

1. (a) % Stimulation = $\left(\frac{\text{uncoupled rate}}{\text{State 4 rate}} \times 100\right) - 100$ (b) % Stimulation to equal state 3 rate = $\left(\frac{\text{State 3 rate}}{\text{State 4 rate}} \times 100\right) - 100$ (c) 0% Stimulation = State 4 rate.

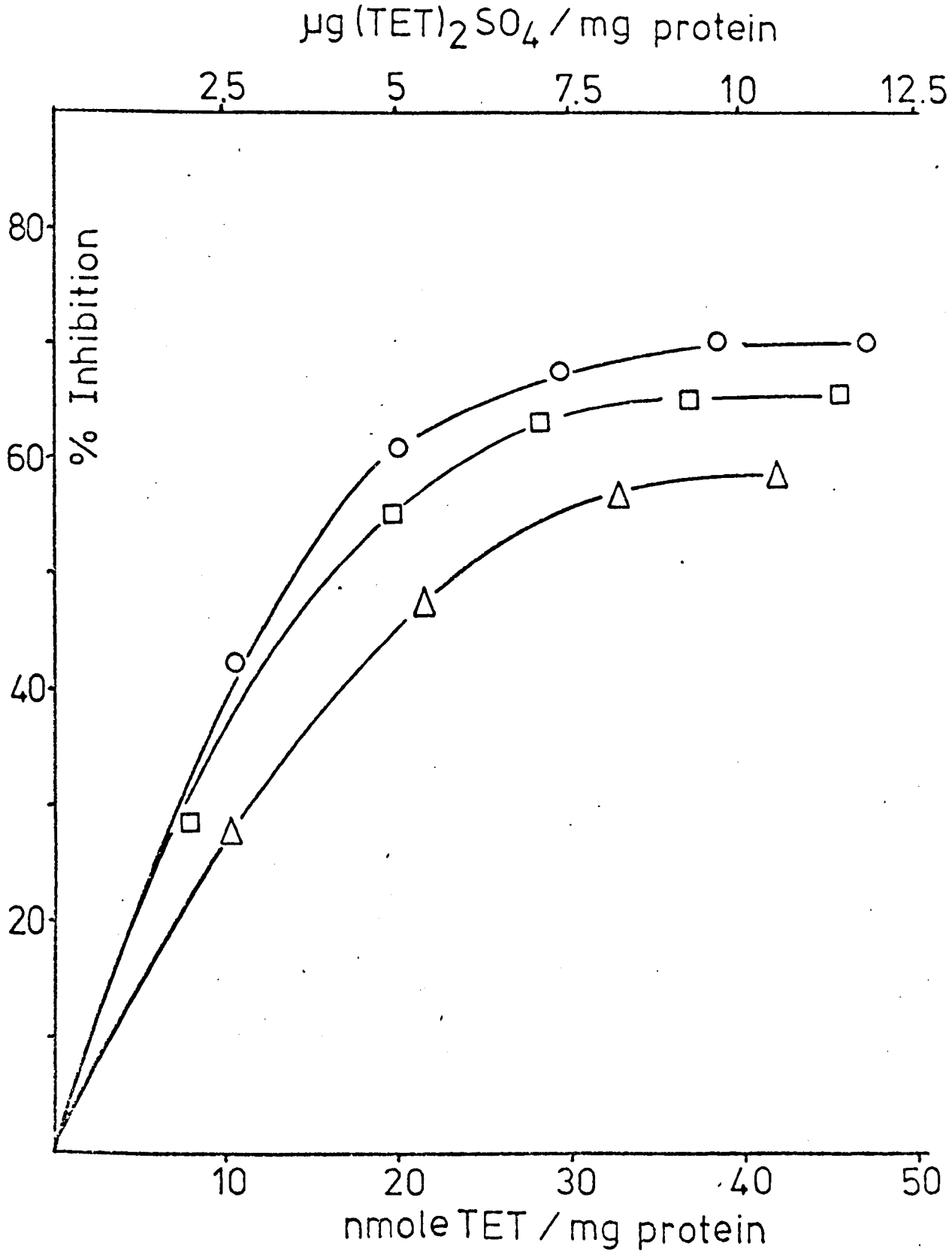
2. These results are obtained from Figures 3.17 and 3.18.

Figure 3.19



The effect of TET on "State" 3 or uncoupler stimulated respiration in mitochondria from S. cerevisiae, strain D22. Cells were grown in the fermenter on 1% (v/v) ethanol and harvested in log phase. Mitochondria were prepared by the snail enzyme procedure.

Figure 3.20



Inhibition of S. cerevisiae mitochondrial "State 3" respiration by TET.

O - O D22. □ - □ D22-CB9. Δ - Δ D22-EC2.

TABLE 3.7.

Inhibition of State 3 respiration in S. cerevisiae mitochondria by TET in the absence of chloride ions.

Strain	Type	Class	mg protein per assay	Average State 3 rate nmole 0_2/min/mg protein	TET concentration at 50% Inhibition		Maximal Values		
					nmole/ mg protein	μM	Maximum % Inhibition	TET concentration on attaining maximum % Inhibition.	
								nmole/mg protein	μM
D22	wild type	-	0.425	128	13.3	2.8	70	37	7.7
D22-CB9	"1799"^{R}	1	1.1	129	19.0	10.5	64	37	20.5
D22-EC2	TET^R	3	1.9	56	24.0	23.0	57	37	36.2

Notes.

1. $$\% \text{ Inhibition} = 100 - \left(\frac{\text{Inhibited Rate - State 4 Rate}}{\text{State 3 Rate - State 4 Rate}} \times 100 \right)$$

2. All TET concentrations are in terms of TET not $(TET)_2SO_4$.

3. These results are an analysis of Figure 3.20.

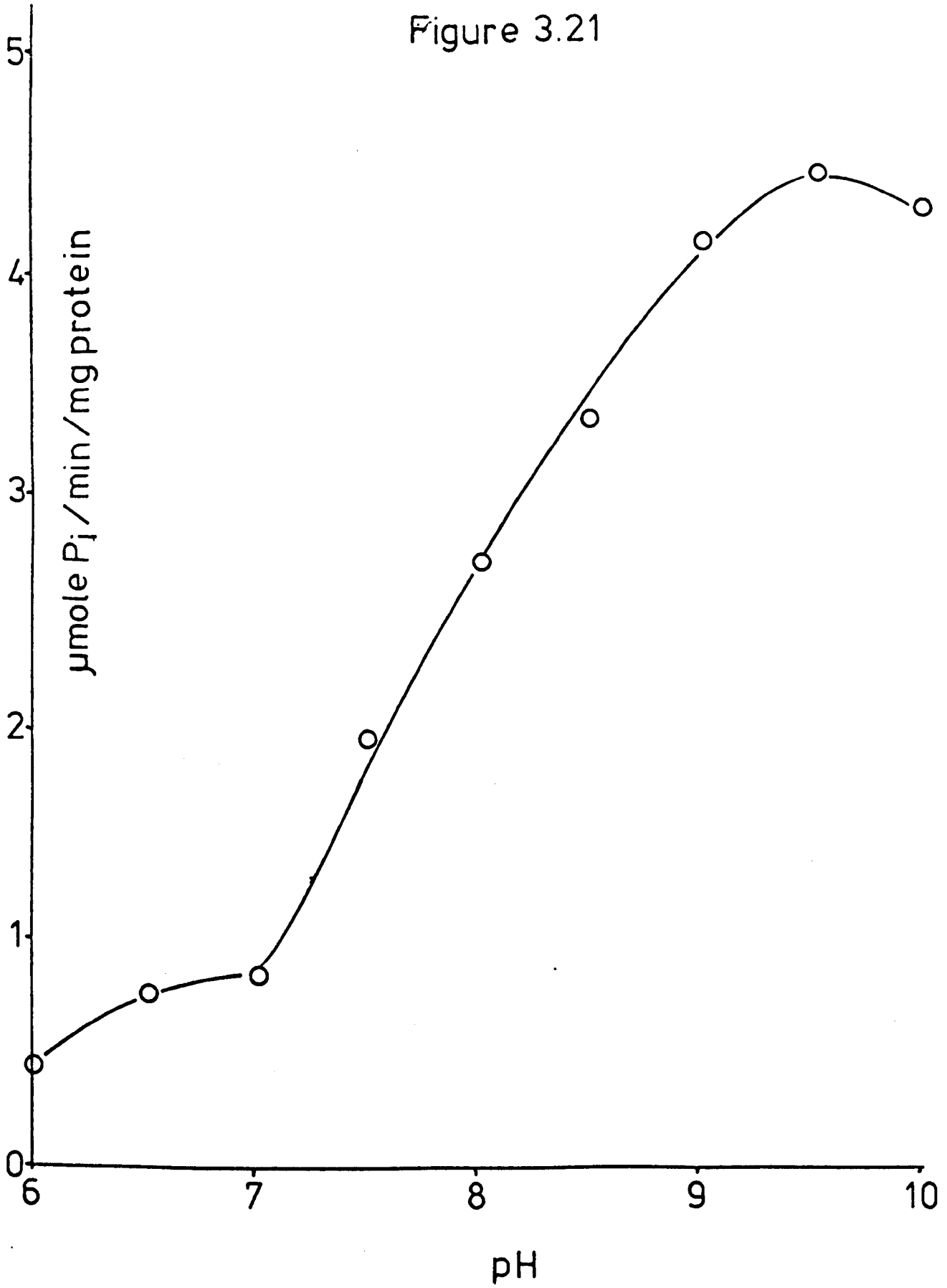
The effects of TET on the respiration of intact mitochondria are complicated by its diverse effects. Its actions on the mitochordrial ATP ase, and on anion transport under the correct conditions, depend on the pH of the assay medium (Coleman and Palmer, 1971). An illustration of the action of TET, at pH 6.5 and in the absence of Cl^-, on the respiratory activity of isolated mitochondria from S. cerevisiae, strain D22, is given in Figure 3.19. High concentration of TET acted like oligomycin and inhibited "State 3" respiration to a rate comparable with that of "State 4". This inhibition could be removed by uncoupler. There was no effect on uncoupler stimulated respiration under these conditions. Experiments to examine the effects of addition of chloride or other anions or variation of pH (to pH 7.4) were not attempted.

The inhibition of "State 3" respiration with increasing TET concentration is plotted in Figure 3.20 for D22, D22-CB9 and D22-EC2 mitochondria. Both the mutants were resistant to TET at low concentrations and in terms of the maximum amount of inhibition that could be obtained (Table 3.7); D22-EC2 was the most resistant. The maximally inhibited "State 3" rates were still greater than the corresponding "State 4" rates in these assays, ie. the State 4 rate represents 100% inhibition in Figure 3.20.

The Effects of Inhibitors on the Mg^{2+}- dependent ATPase Activity of S. cerevisiae Mitochondria.

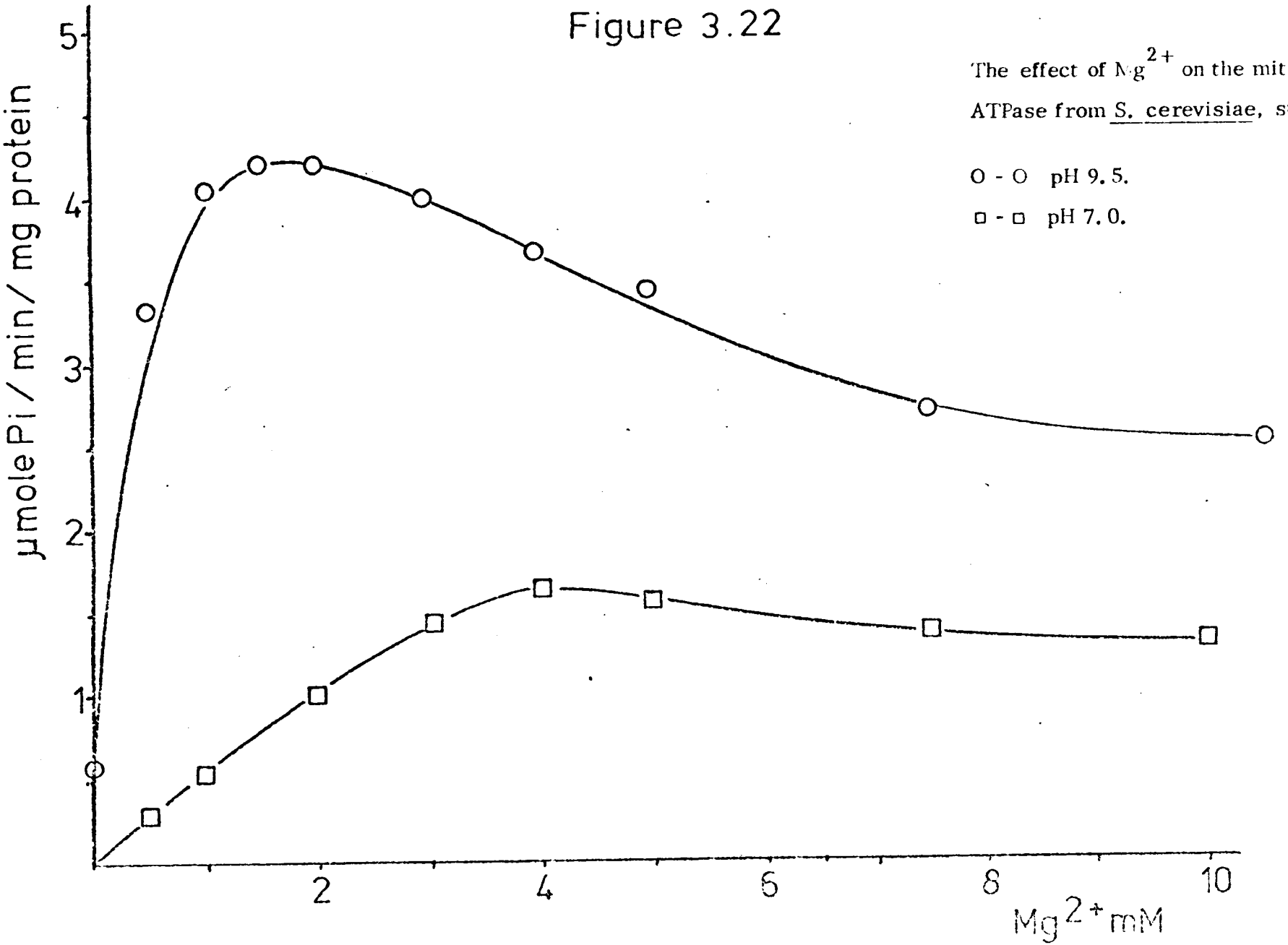
The effects of pH on the mitochondrial, Mg^{2+}- dependent ATPase activity of S. cerevisiae, strain D22, grown on ethanol, are shown in Figure 3-21. Two maxima, at pH 6.5 and at pH 9.5 are observed, in agreement with previous reports (Kovac et al, 1968; Somlo, 1968). The specific activity at pH 9.5 was in the range 4-5 μmole Pi/min/mg protein and this was much greater than the activity at pH 6.5, which was about 0.8 - 1.0 μmole Pi/min/mg protein. These mitochondria were isolated by the Braun shaker procedure and are largely fragmented. Even in intact mitochondria from Saccharomyces sp. it is not necessary for uncoupler to be added for the ATPase activity to become apparent. This enzyme is greatly stimulated by the presence of Mg^{2+} ions although the presence of DNP results in a slight increase in activity at pH values greater than 6.0. Using fresh material, the ATPase activity at pH 9.5 was normally between 5.5 and 7.0 μmole Pi/min/mg protein but on keeping at 0°-5°C overnight this decreased in all cases to 4.0 - 4.5 μmole Pi/min/mg protein. The

Figure 3.21



The effect of pH on the mitochondrial ATPase from S. cerevisiae, strain D22. Cells were grown in the fermenter on 0.5% (v/v) ethanol and harvested in stationary phase. Mitochondria were prepared using the Braun shaker and gradient purified before use.

Figure 3.22

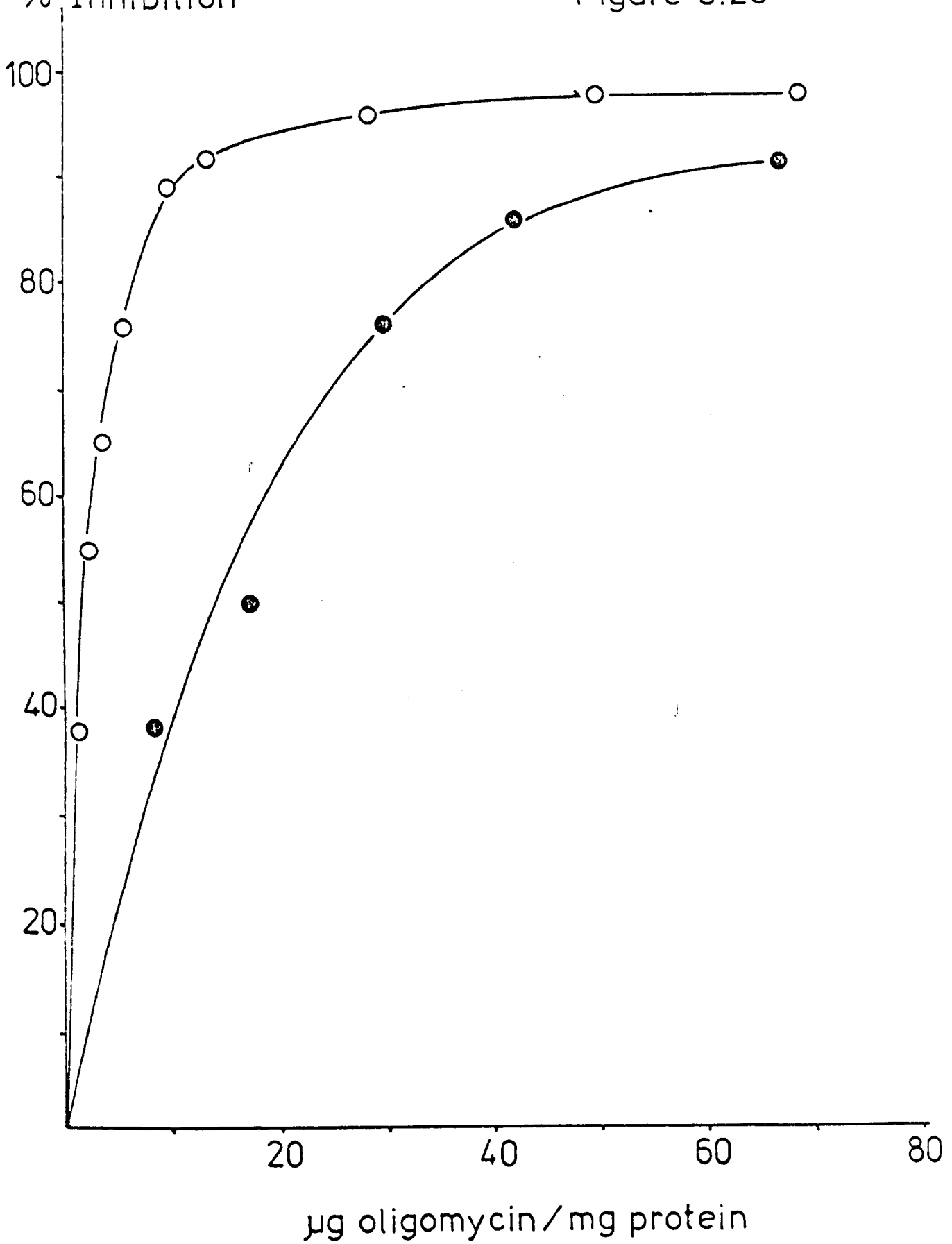


The effect of Mg^{2+} on the mitochondrial ATPase from S. cerevisiae, strain D22.

O - O pH 9.5.

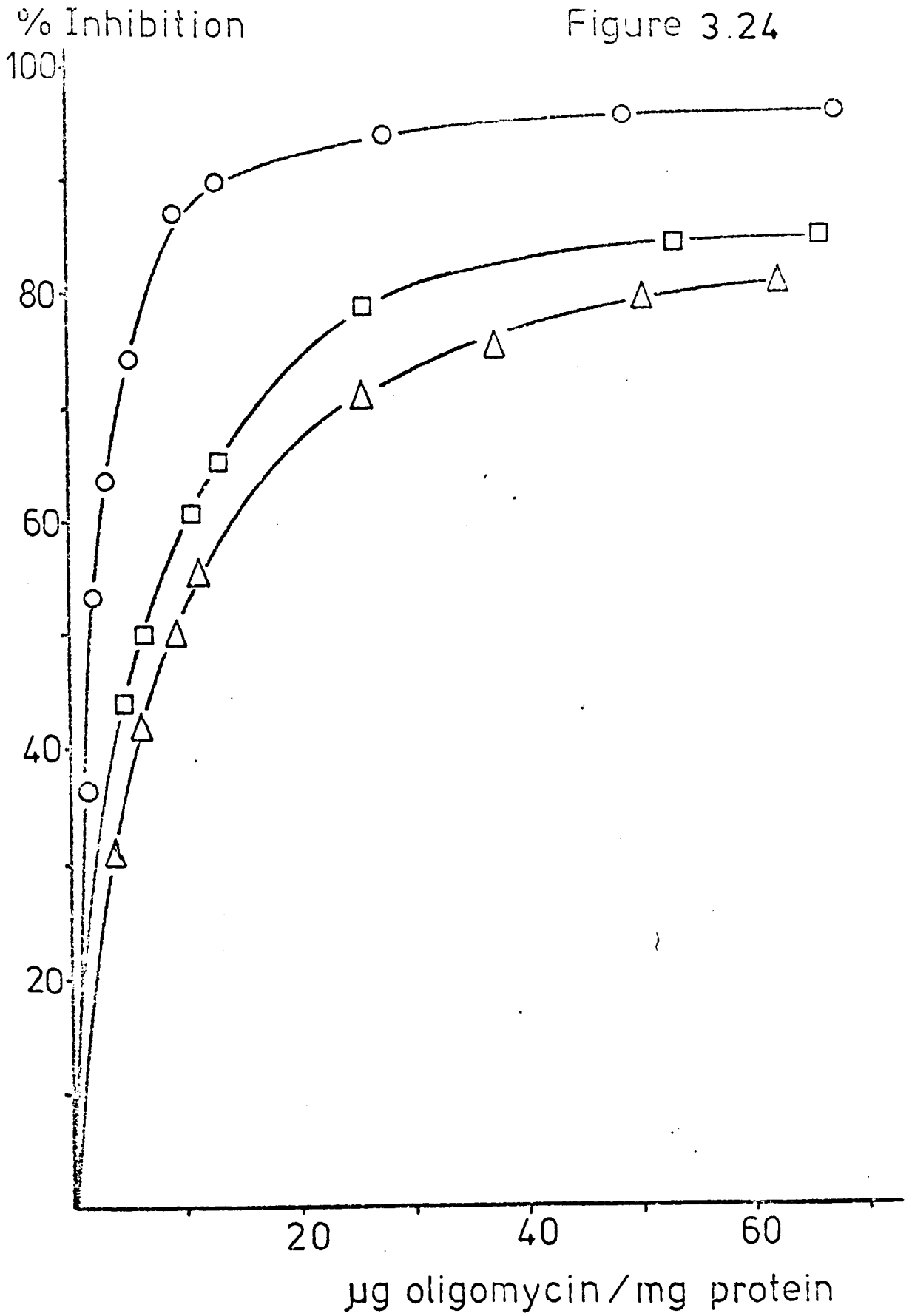
□ - □ pH 7.0.

Figure 3.23



The effect of oligomycin on the mitochondrial ATPase in S. cerevisiae, strain D22.

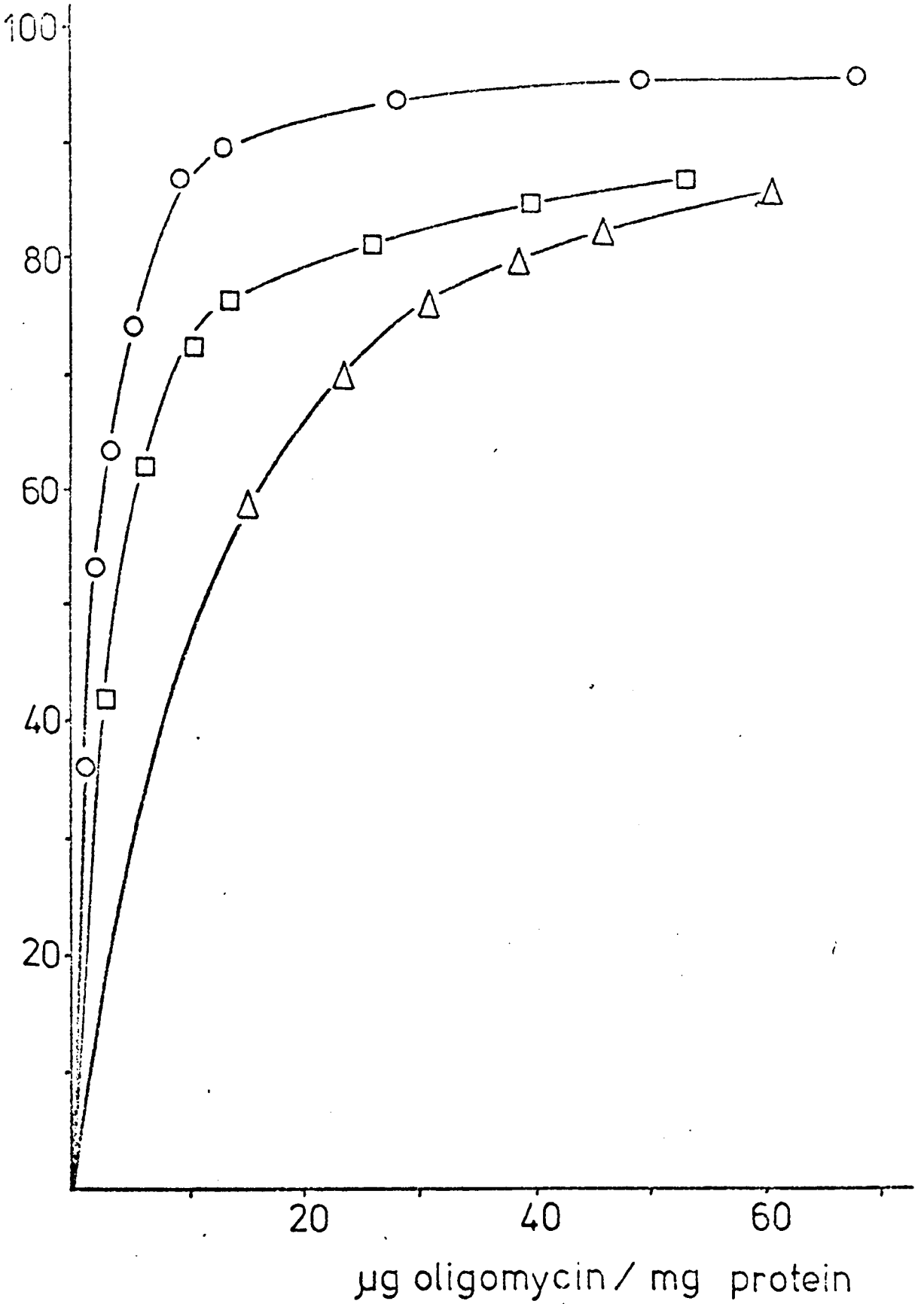
O - O pH 9.5. ● - ● pH 7.0.



The effect of oligomycin on the mitochondrial ATPase at pH 9.5 in S. cerevisiae.

O - O D22. □ - □ D22 - DCS12. Δ - Δ D22 - DCS9.

Figure 3.25



The effect of oligomycin on the mitochondrial ATPase at pH 9.5 in S. cerevisiae.

O - O D22. □ - □ D22-CB19. Δ - Δ D22-CB9.

TABLE 3.8.

Effect of oligomycin on the mitochondrial ATPase from S. cerevisiae.

Strain	Type	Class	pH of Assay	μg oligomycin/mg protein			μg oligomycin/mg protein at 50% Inhibition.
				2	10	50	
				Percentage Inhibition			
D22	wild type	-	9.5	42	86	95	2.5
D22	wild type	-	7.0	10	38	87	15
D22-DCS12	$TTFB^R$	2	9.5	30	60	84	6
D22-DCS9	$TTFB^R$	3	9.5	21	52.5	79	9
D22-CB9	"1799"^{R}	1	9.5	13	47	85	12
D22-CB19	"1799"^{R}	2	9.5	35	74	88	4

Notes.

1. These results are from Figures 3.24 and 3.25

pH profiles of the mitochondrial ATPase activities present in the TTFB^R mutants, D22-DCS12 and D22-DCS9; the "1799"^{R} mutants, D22-CB9 and D22-CB19; and the TET^R strains D22-EC1 and D22-EC2 were approximately same as that of the wild type.

Since the mitochondrial ATPase in S. cerevisiae is Mg^{2+}- dependent, the optimal concentrations of Mg^{2+} were determined at each pH maximum (Figure 3.22). In agreement with Kovac et al, (1968) the optimal Mg^{2+}: ATP ratios were 1:1 at pH 7.0 and 1:2 at pH 9.5.

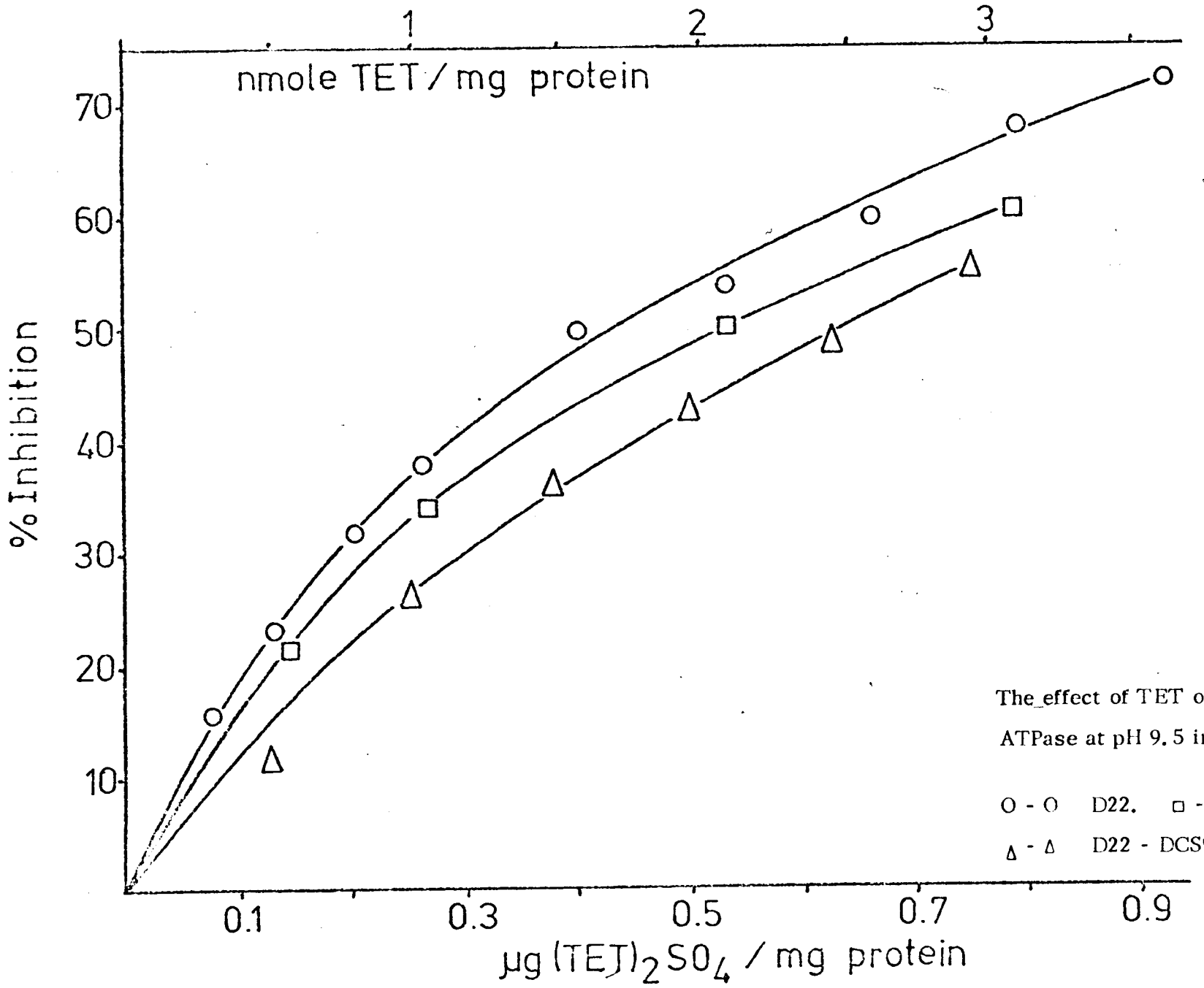
The mitochondrial ATPase of S cerevisiae, strain D22, was more resistant to oligomycin when assayed at pH 7.0 than when assayed at pH 9.5 (Figure 3.23), in agreement with previous reports (Kovac et al, 1968; Somlo, 1968). 50% inhibition was found at 15 µg oligomycin/mg protein at pH 7.0 and at 2.5 µg oligomycin/mg protein at pH 9.5. At high concentrations of oligomycin (50 µg/mg protein) the activities at both pH 7.0 and pH 9.5 were inhibited to around 90% (Table 3.8).

The effects of oligomycin on the mitochondrial ATPase activity, at pH 9.5, of S. cerevisiae, strain D22, and of the TTFB^R strains D22-DCS12 and D22-DCS9, are illustrated in Figure 3.24. The enzymes from the mutant strains were less sensitive to oligomycin inhibition than the wild type activity, 50% inhibition values occurred at 2.5, 6.0 and 9.0 µg oligomycin/ mg protein respectively and further data are given in Table 3.8. This again is evidence that the mutation, specifically to TTFB resistance, may give rise to more widespread effects as a result of a presumed specific change at the uncoupler binding site. Similar results were also obtained for the effects of oligomycin on the mitochondrial ATPase activities of "1799"^{R} mutants (Figure 3.25). Compared to the wild type, the strains D22-CB9 and D22-CB19 are relatively resistant to oligomycin (Table 3.8). The responses of the mitochondrial ATPase activities of the TET^R mutants D22-EC1 and D22-EC2 towards oligomycin were the same as the wild type. This is in line with previous determinations of cross-resistance using plating techniques (Table 2.3).

The effects of TET on the ATPase activities of the S. cerevisiae, D22 wild type and TTFB^R strains, D22-DCS12 and D22- DCS9, are shown in Figure 3.26. Both of the mutants showed some resistance to the inhibitor relative to the wild type (Table 3.9). These results were not anticipated from plating studies (Table 2.4). However in agreement with previous experiments on intact cells (Table 3.3) and on isolated mitochondria (Table 3.7)

the mitochondrial ATPases from the "1799"^{R} strains D22-CB9 and D22-CB19 were less sensitive to inhibition by TET than the wild type enzyme (Figure 3.27). Possibly because it is a "Class 1" mutant D22-CB9 was the more resistant (Table 3.9). This assay of the mitochondrial ATPase has been used, in the case of the, TET^{R} strains D22-EC1 and D22-EC2, to confirm that resistance to TET is expressed at the ATPase level (Figure 3.28). It is noticeable from Table 3.9 that strains that are resistant or cross-resistant to "1799" on plates, ie. D22-DCS9, D22-CB9, D22-CB19 and D22-EC2, seem to be the most resistant to TET. This is further evidence for some association between TET and "1799".

Figure 3.26

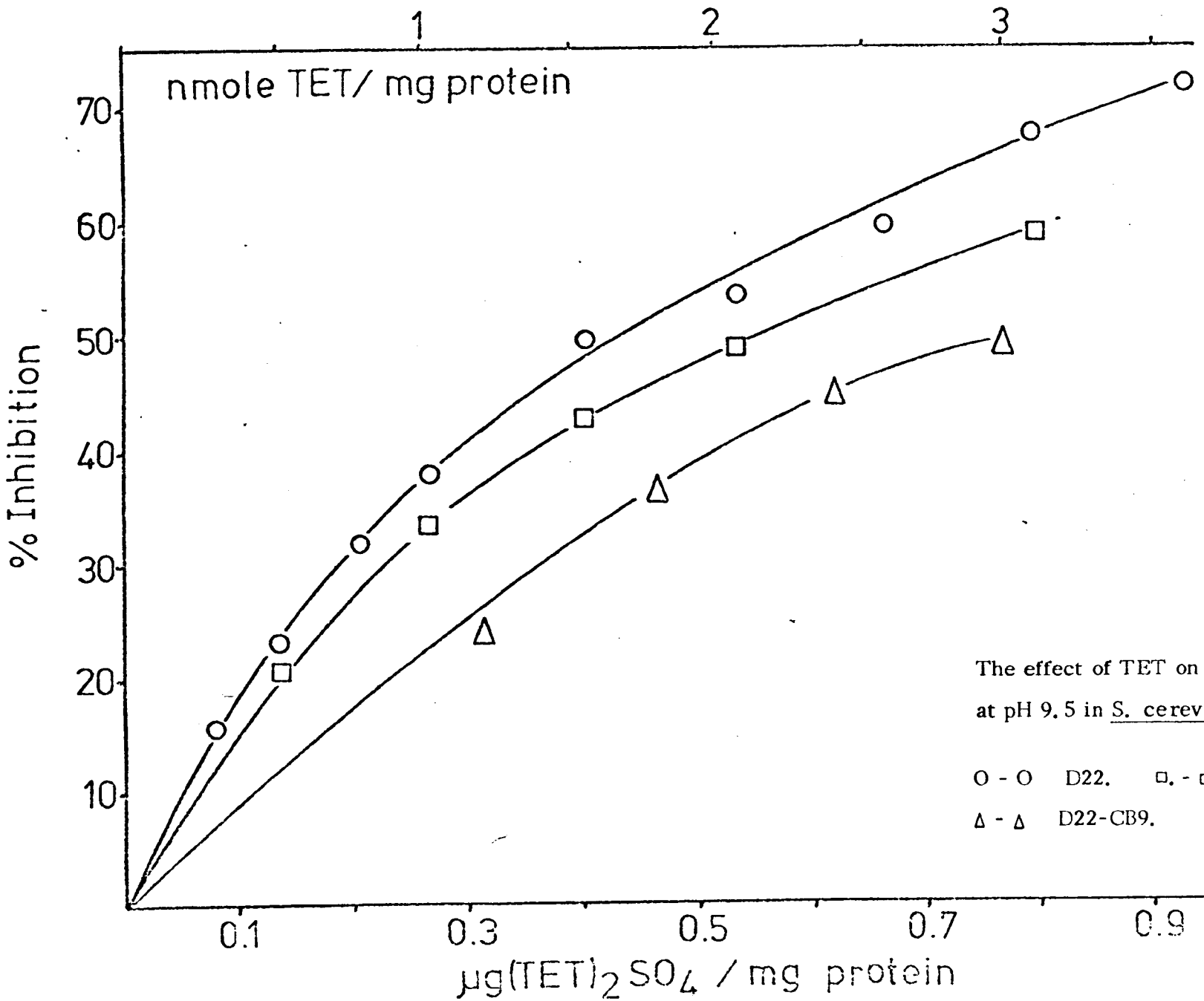


The effect of TET on the mitochondrial ATPase at pH 9.5 in S. cerevisiae.

O - O D22. □ -.□ D22-DCS12.

Δ - Δ D22 - DCS9.

Figure 3.27

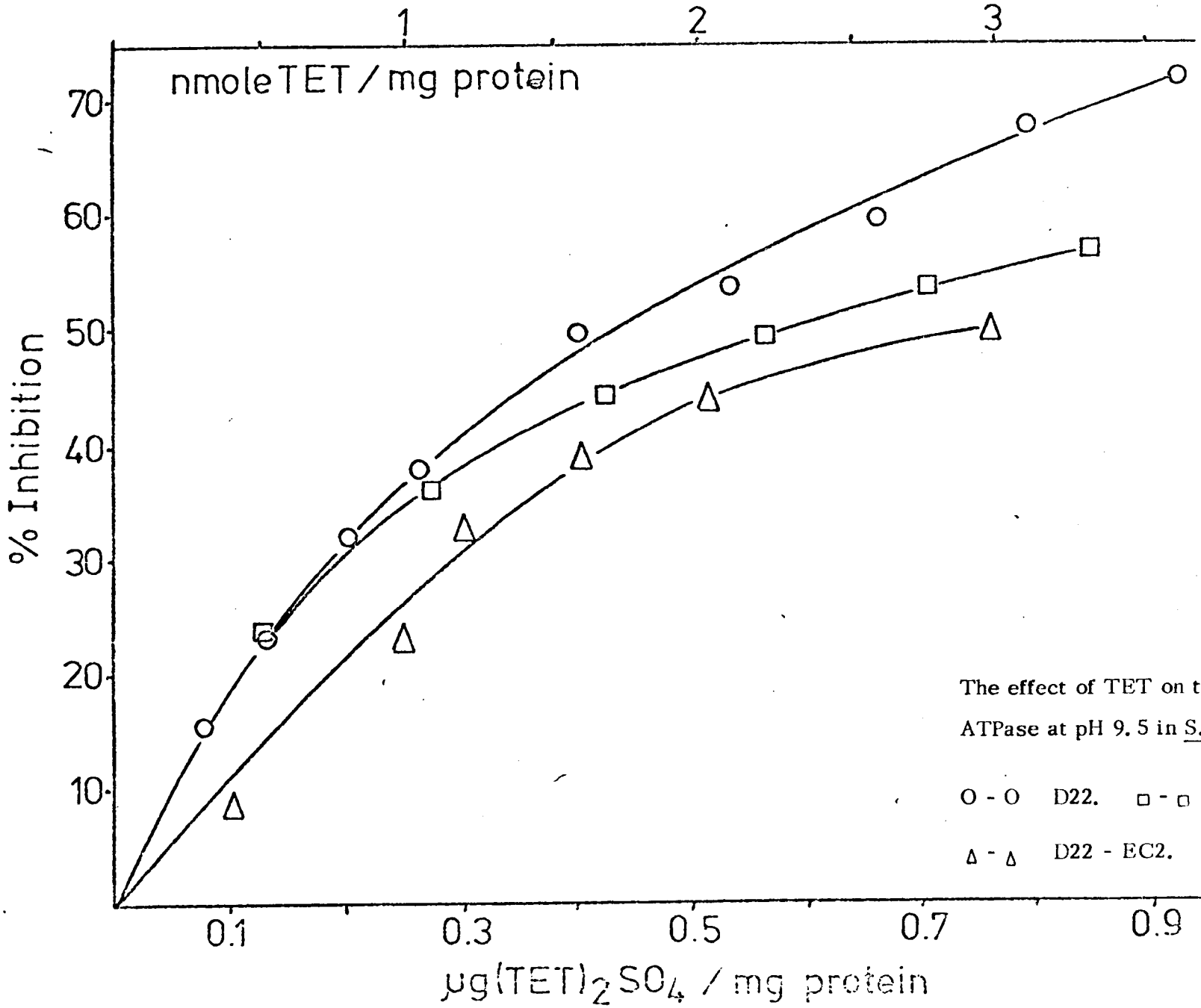


The effect of TET on the mitochondrial ATPase at pH 9.5 in S. cerevisiae.

O - O D22. □ - □ D22-CB19.

Δ - Δ D22-CB9.

Figure 3.28



The effect of TET on the mitochondrial ATPase at pH 9.5 in S. cerevisiae.

O - O D22. □ - □ D22 - EC1.

Δ - Δ D22 - EC2.

TABLE 3.9.

Effect of TET on the mitochondrial ATPase, at pH 9.5, from S. cerevisiae.

Strain	Type	Class	$\mu g(TET)_2SO_4$/mg protein		$\mu g (TET)_2SO_4$/mg protein at 50% Inhibition.
			0.2	0.7	
			Percentage Inhibition		
D22	wild type	-	32	63	0.43
D22-DCS12	$TTFB^R$	2	28	57	0.53
D22-DCS9	$TTFB^R$	3	22	53	0.63
D22-CB9	"1799"^{R}	1	17.5	48	0.8
D22-CB19	"1799"^{R}	2	27	56	0.56
D22-EC1	TET^R	2	30	53	0.6
D22-EC2	TET^R	3	21	49	0.76

Notes.

1. These results are from Figures 3.26 - 3.28.

DISCUSSION.

Uncoupling agents act to uncouple electron transport from the corresponding phosphorylation reaction in oxidative and photosynthetic energy conservation systems. Uncouplers therefore affect energy metabolism in bacteria (Jackson et al, 1968), chloroplasts(Montal et al, 1970; Walker and Crofts, 1970) and mitochondria (Lehninger, 1964: Greville, 1969). All these structures may be characterised by their ability to maintain an electrogenic pH gradient across a specialised membrane as part of the energy coupling process, (Greville, 1969). Uncouplers may increase the proton permeabilities of the mitochondrial inner membrane (Mitchell and Moyle, 1967 a, b) and of artificial membrane structures (Chappell and Haarhof, 1967; Skulachev et al, 1967) and can also affect the permeability properties of other membranes eg. cell membrane (Harris and Pressman, 1967).

The passive permeability of S. cerevisiae is comparatively low, the cells are virtually impermeable to most anions and also to cations (in the absence of substrate) (Rothstein, 1959). Large changes in the permeability of the yeast cell membrane can however be caused by detergents (Armstrong, 1963). If resting yeast cells are provided with a metabolisable substrate, various ions are taken up by specific pathways eg. the "K^+-H^+", "$H_2PO_4^-$" and "Mg^{2+} - $H_2PO_4^-$" systems (Armstrong, 1972). Potassium uptake is a 1:1 exchange with internal protons (Conway and O'Malley, 1946) and there is net movement of both species against large concentration gradients. This exchange mechanism is inhibited by azide or DNP, and there is no evidence for the participation of a Na^+/K^+ - ATPase in this situation in yeast (Cirillo, 1966). The properties of the other ion accumulation systems localised in the yeast cell membrane have been described by Rothstein, (1961). The S. cerevisiae cell membrane is therefore capable of selective permeability and active transport of ions and other molecules.

It has been mentioned that resistance to uncouplers, or indeed to any agent specifically affecting mitochondrial function, may arise through a change in the permeability of the cell membrane such that the agent does not reach its site of action. This is however unlikely in mutants having mitochondrial genetics.

Uncouplers may be expected to have some effect on the ion balance across the yeast cell membrane, particularly that of the "K^+ - H^+" system. It is not impossible that in sensitive strains the cell would be affected as much from the variations in its ionic contents, or from energy depletion in trying to maintain these, as from the decrease in ATP supply brought about by the actions of these agents on the mitochondria.

The results, summarised in Tables 3. 1 and 3. 2, show that mutants of S. cerevisiae, strain D22, isolated by plating techniques, are resistant to the action of uncoupling agents on oxygen uptake at the cellular level. The immediate effects of an uncoupling agent are usually reflected in stimulation of respiration. However it is also necessary to consider the differences between the effects of TTFB and other uncouplers eg. CCCP and "1799". The actions of these agents on cellular ion balance and substrate uptake may be relevant in this context. The uncouplers are acting at the mitochondrial level in stimulating oxygen uptake and reducing ATP synthesis but similarly in trying to maintain ion balance across the cell membrane the expenditure of energy by the cell and consequently the rate of respiration would be increased. TTFB may have some effect on substrate uptake or its availability to the respiratory chain, in order to produce inhibition of respiration. Alternatively, it may be a more effective uncoupler than either CCCP of "1799" and as a result it may induce some kind of respiratory control. Since TTFB was more effective at lower pH values (Figure 3. 2) in inhibiting oxygen uptake; entry into the cell would therefore depend on the non-ionised form of the molecule. Sels, (1969) has shown a similar pH dependence for the action of benzimidazole on S. cerevisiae cells. This chemical inhibits yeast growth and the induced biosynthesis of the functional respiratory chain. All uncouplers will cause depletion of the ATP content of the cell and this will affect synthetic reactions. It is also possible that there is a long term effect of TTFB on protein synthesis due to disorganisation of the mitochondrial membrane-ribosome complex (Bunn et al, 1970; Towers et al, 1973). If TTFB is effective in disorganising the inner mitochondrial membrane this might also be expected to lead to inhibition of respiration. The other uncouplers tested, ie. CCCP and "1799", stimulate oxygen uptake in whole cells and it is concluded that in this case substrate uptake and availability to the respiratory chain are unaffected. The operation of the respiratory enzymes localised in the inner mitochondrial membrane is also uninhibited. Both CCCP and "1799" are more effective at lower pH's and are presumed to

diffuse into the cell in their non-ionised forms. Mutants of S. cerevisiae which were resistant on plates to CCCP or to "1799" were normally resistant to stimulation of respiration by these agents. In the case of the $TTFB^R$ mutants, the Class 2 and Class 3 divisions proposed from cross-resistance studies on plates (Griffiths, 1972), were sometimes disobeyed. This may be evidence in favour of possible long term or other effects of "1799". In any case these studies provide more evidence for a difference between "1799" and other "acidic"uncouplers.

More information on the effects of uncoupling agents on intact yeast cells could be provided by measurements of the external pH and potassium ion concentration during uncoupler addition. There are also different uncoupling agents, apart from the "acidic" types, that may be used. These fall into the categories of weak bases, eg. S13 and tributylamine, and lipid soluble anions and cations (Skulachev et al, 1969a,b). Use of these compounds would also provide more insight into the phenomena of uncoupler permeability and action at the whole cell level.

Tri-alkyl and tri-phenyl tin compounds inhibit energy conservation reactions in mitochondria (Aldridge and Street, 1964) and in chloroplasts (Kahn, 1968). These compounds bind to mitochondria and affect oxidative phosphorylation in the same way as oligomycin (Aldridge and Street, 1970; Byington, 1971). They also mediate the transport of halide or thio-cyanate anions across the inner mitochondrial membrane (Manger, 1969; Selwyn et al, 1970; Stockdale et al, 1970). Tri-ethyl tin can therefore affect oxidative phosphorylation in at least two ways (Rose and Aldridge, 1972) and its actions can also be modified by the pH of the assay medium (Coleman and Palmer, 1971). Recently Harris et al, (1973) have shown that tril-alkyl tin compounds also inhibit the transport of adenine nucleotides across the mitochondrial membrane.

The same considerations that have been applied to the action of uncoupling agents at the whole cell level can also be applied to TET. This compound may affect the ion balance across the cell membrane S. cerevisiae, since it has the ability to mediate halide permeability. TET always inhibited the respiration of intact cells (Table 3. 3) and cells which were resistant on plates were also resistant in this assay. The varying amounts of inhibition, depending on the presence or absence of KCl, are difficult to explain. The presence of Cl^- ions increases the lipid solubility of TET (Rose and Aldridge, 1972) and would therefore be expected to increase the transfer of TET across the cell membrane.

It is assumed that, in the presence of Cl^-, TET gets into the cell as a neutral TET chloride molecule. This may alter ion balances across the cell membrane. The primary effect of TET, once in the cell, is presumed to be the inhibition of oxidative phosphorylation by whatever mechanism. This inhibition will not be as effective in the resistant mutants. In the case of the D22 wild type strain, the presence of Cl^- in the assay medium may facilitate the entry of TET into the cell so that at low concentrations of TET, inhibition of respiration is more pronounced than in its absence. Only in the presence of Cl^- were mutants, which were resistant to TET on plates, less inhibited in respiration by low concentrations of TET than the wild type (Table 3.3). In the absence of Cl^-, the resistance phenomenon in the mutants may lead to secondary effects at low concentrations of TET resulting in the inhibition of respiration. Interaction of TET with the inner mitochondrial membrane could produce inhibition of respiration by any of the mechanisms discussed above. The mutants which show mitochondrial genetics and which are resistant to TET would presumably have a change at the TET binding site in the mitochondrial membrane resulting in a reduced affinity for the inhibitor. Tables 3.2 and 3.3 show that the "1799"^{R} mutants are resistant to TET and that the TET^R mutants are resistant to "1799", in agreement with plating experiments (Griffiths, 1972). This is more evidence that TET and "1799" may have some common feature in their respective modes of action.

None of the TTFB^R mutants are resistant to TET on plates. Again measurements of the variations in H^+ and K^+ concentrations in the external medium during the addition of TET would provide meaningful data.

These studies show that inhibitors and uncouplers which have immediate effects on mitochondrial oxidative phosphorylation have similar effects in intact cells. These actions may be presumed to be primarily at the mitochondrial level but, if the agent in question can influence ion transport phenomena, then other effects at the cell membrane level cannot be immediately discounted. Any effect on ion transport across the cell membrane is presumed to be secondary to the effects of uncouplers or TET on oxidative phosphorylation and/or the properties of the inner mitochondrial membrane, since resistant mutants exhibit mitochondrial genetics.

The study of the properties of yeast mitochondria was hindered owing to the difficulty in producing the relatively intact preparations necessary for the observation of coupled phosphorylation and ion transport pheomena. The isolation of intact mitochondria from Saccharomyces carlsbergensis grown in

batch culture was accomplished by Ohnishi et al, (1966a) using the "snail enzyme" method. Several authors have since described the properties of Saccharomyces mitochondria isolated either by mechanical methods (Balcavage and Mattoon, 1968; Guarnieri et al, 1970) or by modifications of the "snail enzyme" technique (Schuurmans-Stekhoven, 1966; Kovac et al, 1968; Ghosh and Bhattacharyya, 1971). As a result of these studies there has been controversy over the existence of "Site 1" energy coupling in yeast mitochondria (Ohnishi, 1973). On the basis of cross-over studies, Chance (1959) concluded that "Site 1" phosphorylation was present in starved S. cerevisiae. But from EPR measurements, and failure to detect ATP dependent, succinate linked, NAD reduction in sub-mitochondrial particles, Schatz and Racker, (1966) and Schatz et al, (1966) concluded that "Site 1" was absent in this yeast. This conclusion was supported by Ohnishi et al, (1966a), Balcavage and Mattoon, (1968) and Kovac et al, (1968). However Ohnishi, (1970) has since shown that induction of "Site 1" energy coupling in S. carlsbergensis is possible. This conclusion is supported by Mackler and Haynes, (1973). In common with Candida utilis mitochondria (Katz, 1971; Katz et al, 1971; Ohnishi, 1972) a "Site 1" bypass mechanism is present in Saccharomyces under optimal (non-carbon limited) growth conditions. External parameters other than the availability of carbon scource can also affect the occurrence of "Site 1" phosphorylation in Saccharomyces sp. (Light and Garland, 1971). Mitochondria from C. utilis (Ohnishi et al, 1966b) or from Neurospora crassa (Weiss et al, 1970) posses internal and external NADH dehydrogenases. Saccharomyces mitochondria have been shown to be the same in this respect (von Jagow & Klingenberg, 1970). These dehydrogenases are in equilibrium owing to the existence of an ethanol-acetaldehyde couple acting across the inner mitochondrial membrane. Presumably, as is the case for C. utilis, "Site 1" energy coupling connected with the internal dehydrogenase develops in Saccharomyces mitochondria under the correct conditions. Therefore isolated Saccharomyces mitochondria may show different P : O ratios for NAD linked substrates depending on the phase of growth and possibly on the particular species and/or strain of yeast used.

Mitochondria isolated from S. cerevisiae, strain D22, and selected uncoupler and TET resistant mutants all exhibit consistently low P : O ratios (Table 3.4). Since these mitochondria were all prepared from log phase cells, "Site 1" phosphorylation is missing. However, compared with other mitochondrial preparations from log or early stationary phase cells (Ohnishi et al,

1966a; Kovac et al, 1968) the P : O ratios of these mitochondria from strain D22 and the mutants derived from it, were always noticeably less, whatever substrate was used. This may be a strain dependent phenomenon. Although having the same P : O ratios as the D22, wild type, mitochondria those isolated from $TTFB^R$ strains all had lower rates of respiration on all substrates tested. These observations correlate with the lower rates of respiration seen in log phase for $TTFB^R$ mutants grown on ethanol in batch culture (Figures 2.4 and 2.5). It is possible that these results may be associated with the lesser amounts of cytochrome aa_3 found in the cells of these $TTFB^R$ mutants compared with the wild type (Table 2.11). However, the "1799" ^{R} strain, D22-CB9, has approximately the same rates of oxygen uptake on all substrates as strain D22, but much less measureable cytochrome aa_3. Conversely D22-EC2 has almost the same amount of cytochrome aa_3 but the rates of respiration in isloated mitochondria are about 50% of the corresponding wild type rates. These observations are difficult to reconcile with growth curves and growth yields of D22-EC2 (Tables 2.6 - 2.8) and may reflect damage to the mitochondria during the isolation procedure. Alternatively, different assay conditions, eg. higher concentrations of substrate or ADP, may be necessary in order to observe rates of oxygen uptake comparable with those of wild type mitochondria.

Deficiencies in cytochrome aa_3 content and in rates of respiration are associated with specific mutation, by the mitochondrial genome, to uncoupler or TET resistance. Further work is necessary in order to provide more data on the effects of these agents on mitochondrial processes in yeast. Examination of the binding sites of uncouplers (Hanstein and Hatefi, 1974a) and of TET in yeast mitochondria may lead to the possibility that specific changes in protein components can be correlated with variations in the properties of the inner mitochondrial membrane.

Energy dependent reverse electron flow has been observed in C. utilis mitochondria at Site 1 (Garland et al, 1972) and at Site 2 in S. carlsbergensis (Ohnishi et al, 1967). It is not possible to observe ATP-dependent changes in the redox levels of respiratory carriers in yeast mitochondria (Sato et al, 1972), but other partial reactions of oxidative phosphorylation are available for study. Kovac et al, (1968) have concluded that the ATP-Pi exchange activity of intact S. cerevisiae is similar to that in animal mitochondria (Boyer, 1967). This method has been used to assay oligomycin resistance, at the mitochondrial level, in OL^R mutants (Griffiths et al, (1972).

Mitochondria organise the transport of anions and cations across their inner membrane, dependent on the energy conservation process (Chance et al, 1967; Cockrell et al, 1967; Lehninger et al, 1967; Chappell, 1968; Greville, 1969; Mitchell and Moyle, 1969). The permeability of S. cerevisiae mitochondria towards K^+ or Ca^{2+} ions, together with the energy dependence of cation uptake, has been studied by Kovac et al,(1972) and Balcavage et al, (1973) respectively. Measurements of the cation fluxes across the mitochondrial membranes of uncoupler resistant S. cerevisiae mutants upon treatment with uncoupling agents would provide useful data.

Brunt et al, (1971) provided evidence for the existence of substrate anion porter systems in the inner membrane of S. cerevisiae mitochondria and subsequently reported the existence of specific carriers for di- and tri-carboxylic acids (Spencer et al, 1971). These observations have been extended by Linnane and co-workers (Perkins et al, 1972) who have also shown that, similar to the respiratory chain and ATPase complexes in yeast mitochondria, these porter systems are amenable to physiological and genetic manipulation. All the protein components of these anion carriers are coded for by nuclear genes and synthesised on cytoplasmic ribosomes (Perkins et al, 1973b). Therefore these carrier systems are present in the mitochondria in anaerobic and/or "petite" yeast cells (Kolarov et al, 1972a; Linnane and Haslam, 1970).

S. cerevisiae mitochondria provide a good system for resolution of the adenine nucleotide transporter of the inner membrane (Klingenberg, 1970). This translocase was initially characterised in S. cerevisiae by Ohnishi et al, (1967). Haslam et al, (1973a) have concluded that products of the mitochondrial protein synthesising system are required for normal functioning of this adenine nucleotide porter. The involvement of the two genetic systems has led to the characterisation of nuclear mutants affected in nucleotide exchange capacity (Kolarov et al, 1972a) and also to examination of the effects of the "petite" mutation on this process. Strains of S. cerevisiae resistant to bongkrekic acid have been isolated as nuclear mutants by Lauquin et al, (1973) and Perkins et al,(1973a); and certain of the cytoplasmic, TET^R mutants of S. cerevisiae, strain D22 are also cross resistant to this inhibitor (Cain et al, 1974).

Complementary to these studies there has been a considerable amount of work on the development of oxidative phosphorylation in S. cerevisiae mitochondria undergoing respiratory adaptation. The effects of nuclear or cytoplasmic mutation(s) resulting in deficiencies in mitochondrial components, eg. lack of specific cytochrome(s), on the energy conservation processes have been studied in detail and will be discussed later.

Measurements of the effects of uncouplers on oxidative phosphorylation in isolated mitochondria from S. cerevisiae, strain D22 and selected mutants, were done primarily to confirm the indications of genetic analyses, that resistance to these agents is expressed at the mitochondrial level. The results are shown in Tables 3.5 and 3.6 for CCCP and "1799" respectively. Cells which were resistant to uncouplers on plates were resistant in these assays.

However, the results correlated better with the effects of uncouplers on the rate of respiration of the corresponding intact cells (Tables 3.1 and 3.2). This was especially true for the action of "1799" on mitochondria from the $TTFB^R$ mutant D22-DCS9

Measurements of the effectiveness of uncoupling agents on the energy conservation process in isolated mitochondria have been used by many workers to provide an insight into the mechanism of oxidative phosphorylation. The "chemical" hypothesis assumes the existence of a "high-energy intermediate" with which uncouplers are presumed to interact (Slater, 1966). There have therefore been experiments to determine the stoichiometry of uncoupling in mitochondria (Margolis et al, 1967; Wilson, 1969; Kaplay et al, 1970; Wilson et al, 1971). According to the "chemiosmotic" hypothesis, uncouplers affect energy conservation reactions in mitochondria by inducing proton permeability in the inner membrane (Mitchell, 1966, 1968). This leads to the conclusion that uncouplers must also traverse the membrane. Evidence for this has come from experiments on lipid bilayers (Liberman and Topali, 1968). Apart from the monomolecular proposal of Mitchell, other mechanisms to account for the uncoupler mediated transport of protons across membranes have been proposed (Finkelstein, 1970). Models based on the uptake of uncoupler anions by the mitochondrial substrate porter systems have been put forward (van Dam and Slater, 1967; van Dam and Kraayenhof, 1969), and these may not directly involve carriage of protons across the inner membrane by the uncoupler. There is a large range of compounds which under the correct conditions can uncouple oxidative phosphorylation. These may be classified as proton carriers, either weak acids, eg. TTFB, CCCP, DNP or weak bases, eg. S13, TBA; or lipid soluble anions or cations, eg. TPB^-, DDA^+, which act by collapsing the membrane potential. It is also significant that the compound DCA, which is an alkylating agent, will also act as an uncoupler (Skulachev et al, 1969a,b). There is a correlation between the efficiency of uncoupling in mitochondria and the increase in proton conductance brought about by the action of the uncoupler on lipid bilayers (Liberman et al, 1969; Bakker et al, 1973).

The effects of uncouplers on mammalian mitochondria are to increase the rate of "State 4" respiration, decrease phosphorylation in "State 3", and to induce Mg^{2+}-dependent ATPase activity. Uncouplers have little or no effect on the ATPase in yeast mitochondria (Kovac et al, 1968). All these effects can be utilised

for kinetic assays of the effects of uncouplers and this has led to somedis-crepancies in comparing results (Wilson, 1969, Kaplay et al, 1970). The accuracy of measurements of uncoupler efficiency on mitochondrial oxygen uptake is influenced by the type andconcentration of the uncoupler and also by the concentration of mitochondrial protein in the assay (Nicholls and Wenner, 1970). Margolis et al, (1967) have concluded that uncouplers are highly mobile in the membrane and have a high affinity for actively functioning coupling sites only. Therefore the amount of uncoupler necessary to produce a given degree of uncoupling depends on the rate of generation of the "high-energy intermediate" ie. on the initial coupling efficiency and on the rate of respiration (Tsou and van Dam, 1969). Using the stimulation of "State 4" respiration in mammalian mitochondria as an indication of uncoupling efficiency, 50% uncoupling was found with FCCP at about 5×10^{-11} nmoles/mg protein (Margolis et al, 1967) and with S13 at about 4×10^{-11} nmoles/mg protein (Kaplay et al, 1970). The uncoupler SF 6847, is also effective in this range (Muraoka and Terada, 1972). Assuming in the case of yeast mitochondria that "50% uncoupling" occurs when the "State 4" rate has been stimulated to equal half of the "State 3" rate then the concentrations of uncouplers necessary to affect yeast mitochondria are higher than those which give rise to corresponding effects in mammalian mito-chondria. For mitochondria from S. cerevisiae, strain D22 and uncoupler resistant mutants "50% uncoupling" is given by CCCP in the range 2.5×10^{-10} - 2×10^{-9} nmoles/mg protein, and by "1799" in the range 5×10^{-11} - 8×10^{-9} nmoles/mg protein (Tables 3.5 and 3.6). The uncouplers, FCCP, S13, CCCP and TTFB are highly effective in contrast to "classical" uncouplers, eg. DNP.

These results may be interpretable in terms of the chemical hypothesis and Muraoka and Terada, (1972) have concluded that the uncoupler, SF 6847, may in-teract with the energy conservation site in a direct way by either nudeophilic attack or by formation of a charge transfer complex. Other potent uncouplers, eg. CCCP or S13, have similar nucleophilic groups in their structures (Williamson and Metcalf, 1967) and may interact similarly with the energy coupling sites. A kinetic model of uncoupler action has been proposed by Nicholls and Wenner, (1972) in which the observed stoichicmetry of uncoupler does not reflect chemical binding. There is no theoretical need for uncouplers to be bound to energy conservation assemblies in any specific ratio, and unlike inhibitors, uncouplers may act catalytically.

Using equilibrium methods, Hanstein and Hatefi (1974a) have found that a stoichometric amount of uncoupler (NPA) binds to mitochondria. The stoichiometry between the concentration of respiratory carriers, the amounts of phosphorylation enzymes and the number of uncoupler binding sites suggests molecular interactions rather than electrochemical effects. It is also significant that all the other uncouplers that were examined eg. S13,CCCP, interacted with the same binding site. Genetic analyses of $TTFB^R$ mutants, together with cross-resistance studies also suggest the existence of a definite binding site for uncouplers in the inner mitochondrial membrane (Griffiths, 1972). Trinitrophenol is a membrane impermeable uncoupling agent (Hanstein and Hatefi, 1974b), hence facilitation of trans-membrane proton equilibration may not determine the degree of uncoupling. This provides further evidence that uncoupling results from the interaction of uncouplers with specific sites on the inner mitochondrial membrane and shows that these are more accessible from the matrix side. Further studies on the uncoupler resistant mutants of S. cerevisiae, strain D22 using uncouplers capable of photoaffinity labelling may yield more information on the protein components of the uncoupler binding sites present in mitochondria.

Yeast cells which were resistant or cross resistant to TET on plates were also resistant at the mitochondrial level (Table 3.7). The inhibition of ADP stimulated respiration by TET was assayed and may be due to effects on phosphorylation or on adenine nucleotide exchange as discussed previously. An effect of phosphate concentration on this type of assay has recently been noted (Dawson and Selwyn, 1974) but this does not change the interpretation of these results. In agreement the mitochondrial ATPase in TET^R mutants is also more resistant to inhibition by TET than the wild type activity. The existence of single mutations in the mitochondrial genome which may affect both the adenine nucleotide translocase and the ATP synthetase complex needs to be proved. If structural components of these two systems are associated in some way this would have implications regarding the phosphorylation process. The characteristics of the mitochondrial, Mg^{2+}-dependent ATPase of S. cerevisiae, strain D22, grown to stationary phase on ethanol, compare well with previous results obtained by other workers (Kovac et al, 1968; Somlo, 1968; Watson et al, 1970, 1971). The specific activity, pH profile, Mg^{2+}-dependence and sensitivity to oligomycin are in line with these observations. These experiments were done on mitochondria prepared by the Braun shaker method and these must be presumed to be fragmented. However, even on relatively intact mitochondria prepared using snail enzyme, uncouplers had very

little effect on the ATPase activity, which was almost totally lacking in the absence of Mg^{2+}. Mitochondria from all Saccharomyces sp. have this in common, in contrast to intact mammalian mitochondria which have an obligatory requirement for uncoupler addition in order to stimulate the latent ATPase. All preparations of mitochondria used in the ATPase assays were gradient purified in order to remove any lipid etc. adhering to the membranes. This lipid interferes especially in assays of the effect of oligomycin on the ATPase since it will bind oligomycin giving rise to systematic errors.

The OL^R mutant, S. cerevisiae D22-A21 (Avner and Griffiths, 1970, 1973a, b) has a mitochondrial ATPase with an altered pH profile; with an increase in specific activity at pH 6.5, relative to that of the wild type (R. Houghton, personal communication). In contrast, none of the $TTFB^R$, "1799"^{R} or TET^R mutants examined here had any significant difference in specific activity or in pH profile to that of the wild type enzyme. These observations do not prove that variations of this type cannot occur, but they are considered unlikely except in the case of TET^R mutants (assuming that the resistance mutation exerts a primary effect on the ATPase). The existence of two pH optima in the case of the S. cerevisiae mitochondrial ATPase is still open to discussion. They may be due to two different enzymes or to a single enzyme (Kovac et al, 1968). It can be postulated that the activity at pH 6.5 is due to a reversal of the ATP synthesis enzyme, whereas that at pH 9.5 may have other functions in synthetic reactions in the cell (Kovac and Kuzela, 1966).

Direct evidence for the involvement of the mitochondrial ATPase in oxidative phosphorylation was provided by Pullman et al, (1960). These workers isolated from bovine heart mitochondria, a soluble ATPase which could function as a coupling factor (F_1) in correspondingly depleted submitochondrial particles (Penefsky et al, 1960). This F_1-ATPase was shown to be a large, spherical molecule (Fernandez-Moran et al, 1964; Penfsky and Warner, 1965). When bound to the membrane, F_1-ATPase is the site of ATP synthesis and of the various nucleotide exchange reactions catalysed by the mitochondrion (Fessenden and Racker, 1966; Hinckle et al, 1967). This enzyme is identical with the "inner membrane spheres", visualised by negative staining techniques, and electron microscopy (Kagawa and Racker, 1966; Racker and Hortstman, 1967). The F_1-ATPase consists of at least five subunits (Knowles and Penefsky, 1972) and an ATPase inhibitor protein has also been purified from bovine heart mitochondria (Pullman and Monroy, 1963). This inhibitor, which is absent from most other types of mitochondria, including those from S. cerevisiae (Tzagoloff, 1971a),

may be present as a sixth subunit of the F_1-ATPase as suggested by Senior (1973). No exact subunit stoichiometry can yet be stated, although the molecular weight of the complex lies between 347, 000 (Knowles and Penefsky, 1972) and 360, 000 (Lambeth et al, 1971).

The soluble F_1-ATPase has many properties which differ from those of the membrane bound enzyme eg. cold lability and in-sensitivity to inhibitors such as oligomycin, DCCD or mercurials (Tzagoloff et al, 1968a; Kopaczyk et al, 1968). Preparations of a soluble, oligomycin sensitive enzyme have been obtained but these are only soluble in the presence of detergent, contain lipids and may also require added phospholipid in order to express ATPase activity. Particle sizes range from the relatively large aggregates seen in the CF_o-F_1 preparation of Kagawa and Racker, (1966) and the OS-ATPase of Tzagoloff et al, (1968a, b) to the more dispersed system of Swanljung and Frigeri, (1972) and that of Tzagoloff and Meagher, (1971) from yeast mitochondria. None of these oligomycin-sensitive ATPase preparations is able to catalyse adenine nudeotide exchange reactions or synthesise ATP.

The preparation CF_o-F_1 can be incorporated into phospholipid vesicles with "hydrophobic membrane proteins". These reconstituted systems can catalyse ATP-^{32}Pi exchange and ATP-driven ion translocation (Kagawa, 1972; Kagawa et al, 1973). The idea that the mitochondrial ATPase may also function as a proton translocase was originally put forward by Mitchell (Mitchell, 1961). ATP hydrolysis will generate a membrane potential and/or pH gradient due to proton translocation across the mitochondrial inner membrane (Mitchell, 1968; Moyle and Mitchell, 1973) and also in reconstituted lipoprotein vesicles (Jasaitis et al, 1972; Skulachev, 1972). These reconstituted vesicles are anisotropic since OSCP and CF_o-F_1 are added after the vesicles are formed. Similar vesicles inlayed with cytochrome oxidase, and anisotropic with respect to cytochrome c, form a membrane potential on the oxidation of ascorbate plus TMPD. Such preparations may also catalyse ion translocation (Hinkle et al, 1972). Using vesicles containing all components, net ATP synthesis on oxidation of ascorbate plus TMPD is demonstrated (Racker and Kandrach, 1971, 1973). Such evidence in favour of the chemiosmotic hypotheses (Mitchell, 1961, 1966, 1968; Greville, 1969) is difficult to reconcile with the observations of Hanstein and Hatefi, (1974a, b). It is possible however that specific uncoupler binding sites may exist in such reconstituted systems. It would be of interest to examine the effect of TNP on these vesicles (which are oriented in the same fashion as submitochondrial particles (Kagawa, 1972).

S. cerevisiae OS-ATPase (Tzagoloff, 1969 a, b, 1970, 1971a) resembles the bovine heart preparations (Kagawa and Racker, 1966; Bulos and Racker, 1968; MacLennan and Tzagoloff, 1968) in being composed of F_1-ATPase together with OSCP and a membrane factor. The yeast F_1-ATPase (molecular weight 340,000) consists of five subunits (Tzagoloff and Meagher, 1971) and the membrane factor has four components (Tzagoloff and Meagher, 1971; Tzagoloff and Akai, 1972; Tzagoloff et al, 1973). The complete OS-ATPase has a molecular weight of 468,000. The results of Stekhoven et al, (1972) for the bovine heart mitochondrial ATPase would also indicate a similar subunit structure (cf. Senior, 1973). The soluble ATPase from Streptococcus faecalis (Abrams, 1965) also interacts with the membrane through a protein (nectin) similar to OSCP. This protein renders the soluble ATPase sensitive to energy transfer inhibitors, eg. DCCD, in the presence of the membrane (Baron and Abrams, 1971).

The sites of action of oligomycin or DCCD appear to be in the membrane factor present in oligomycin-sensitive ATPase preparations (Kagawa and Racker, 1966; Schatz et al, 1967; Knowles et al, 1971). Oligomycin does not form a covalent bond with any component of the mitochondrial membrane, therefore detailed examination of its site of action has not been possible although its molecular structure is now known as a result of X-ray analysis (von Glehn et al, 1972). There is however some evidence that oligomycin acts through an association with phospholipids in the inner membrane (Pitotti et al, 1972). DCCD resembles oligomycin in its effects on energy linked reactions in mitochondria and it also forms a covalent bond with a specific protein in the inner membrane of intact preparations of bovine heart mitochondria (Cattell et al, 1971). The site of action of DCCD has been further localised to one sub-unit of the bovine heart OS-ATPase (Stekhoven et al, 1972). This component is a proteolipid between 10,000 and 14,000 in molecular weight. The great effectiveness of DCCD as an inhibitor may be due to its ability to penetrate into a hydrophobic environment (Abrams and Baron, 1970). It is probable however that DCCD may not bind covalently to the yeast inner mitochondrial membrane (Broughall, 1973).

The characteristics of the yeast and bovine heart mitochondrial OS-ATPases are quite similar. However, higher concentrations of oligomycin (or rutamycin) are required to inhibit the ATPase activity of the isolated yeast enzyme (Tzagoloff, 1969 a, b) and also the enzyme in intact mitochondria (Ohnishi et al, 1966; Kovac et al, 1968; Somlo, 1968). When yeast F_1-ATPase is bound to F_1-depleted bovine

heart submitochondrial particles the reconstituted hybrid ATPase has the same inhibition characteristics as the native bovine heart enzyme (Schatz et al, 1967). This observation emphasises the importance of the membrane factor in modifying the properties of the F_1-ATPase, in all mitochondria.

Significant variations were found between the response to oligomycin of the S. cerevisiae, strain D22, mitochondrial ATPase activity at pH=9.5 in ethanol grown cells and the response of the corresponding enzyme in $TFFB^R$, "1799"^{R} and TET^R mutants (Table 3.8). The variations in 50% inhibition values are not great relative to those found for the series of OL^R mutants of S. cerevisiae [where for the mitochondrial ATPase of strain D22-A21, a 50% inhibition value of 20 µg oligomycin/mg protein was obtained (R. Houghton, personal Communication)]. The most significant differences are seen in the maximal inhibition levels at pH 9.5 which, for all the uncoupler resistant mutants, were less than the wild type. No assays were done at pH 6.5. The TET^R mutants had mitochondrial ATPase activities at pH 9.5 with essentially wild type characteristics of inhibition by oligomycin. In any case, uncouplers produced little or no stimulation of ATPase activity at pH 9.5.

These results may be rationalised by postulating that mutation to uncoupler resistance may also affect the oligomycin binding site such that the characteristics of interaction with the antibiotic are altered or the cooperative response produced by oligomycin binding is affected. This change is most likely in a protein component of the membrane or ATPase complex since there is no variation in the fatty acid makeup of the cell in any of the mutants tested (Chapter 5). The particular subunit(s) affected must be cytoplasmically determined (see Chapter 1) except in the case of the "1799"^{R} strain, D22-CB9 which is a Class 1 mutation and has nuclear genetics.

The mitochondrial ATPase of S. cerevisiae is subject to changes in the external environment as well as in the genotype of the cell. It is subject to repression by glucose and is also depressed by anaerobiosis. In anaerobically grown, glucose repressed cells, of S. cerevisiae the ATPase activity decreases to approximately 20% of that of aerobically grown, derepressed cells (Watson et al, 1970, 1971). The sensitivity to inhibition by oligomycin is also less.

The biosynthesis of mitochondrial components in S. cerevisiae can be studied under conditions of derepression from growth on a relatively high concentration of glucose. Schatz, (1968) and Kovac and Weissova, (1968) observed that "petite" mutants of S. cerevisiae synthesise a cold-labile F_1-ATPase,

suggesting that this part of the ATPase complex was nuclear coded and synthesised cytoplasmically. This interpretation was supported by inhibitor studies using CAP and CHX (Tzagoloff, 1969b; Tzagoloff et al, 1972) which showed that all five subunits of the S. cerevisiae F_1-ATPase were translated and assembled independently of mitochondrial protein synthesis, and similarly for OSCP (Tzagoloff, 1970). The membrane factor necessary for the conferral of oligomycin (or rutamycin) sensitivity is synthesised on mitochondrial ribosomes, (Tzagoloff et al, 1973). These proteins are necessary for the correct integration of F_1 and OSCP into the ATPase complex in the mitochondrial membrane. Kim and Beattie, (1973) have also shown that both cytoplasmic and mitochondrial protein synthesis is necessary at the same time for the expression of mitochondrial ATPase activity. In S. cerevisiae, cytoplasmic mutants which are resistant to oligomycin, this resistance is defined by the membrane factor of the mitochondrial ATPase complex (Shannon et al, 1973). Therefore mitochondrial DNA can direct the synthesis of a membrane component which influences the activity of the mitochondrial ATPase. A significant variation in the properties of one or more of the cytoplamically synthesised subunits of the mitochondrial ATPase may also be present in the series of OL^R mutants described by Avner and Griffiths, (1970, 1973 a, b). The biosynthesis of mitochondrial components independently of cytoplasmic protein synthesis during conditions of derepression and/or as a result of an anaerobic to aerobic transition has also been described by other workers (Groot et al, 1972; Mason et al, 1972; Murray and Linnane, 1972).

These studies have led to the examination of the development of energy transfer reactions in S. cerevisiae under similar conditions. Promitochondria from anaerobically grown S. cerevisiae cells lack all functional cytochromes but retain an oligomycin-sensitive ATPase activity (Criddle and Schatz, 1969; Watson et al, 1971) and also catalyse an uncoupler sensitive ATP-^{32}Pi exchange activity (Groot et al, 1971). This is abolished by the "petite" mutation and emphasises the involvement of mitochondrially synthesised components in energy transfer reactions which occur even in promitochondria from anaerobic, glucose repressed S. cerevisiae cells.

The gross effects of the cytoplasmic petite mutation on the structure and functions of S. cerevisiae mitochondria are well known. More specific deficiencies in one or more of the components of oxidative phosphorylation can be brought about by nuclear mutations. Those resulting in a lack of some respiratory chain components have been discussed. The so called "oxidative phosphorylation mutant" p_9 or op_1 (Kovac et al, 1967a; Kovac and Hrusovoka, 1968)

is a strain with a defective adenine nucleotide translocase and high ATPase activity (Beck et al, 1968; Kolarov et al, 1972 b). The mitochondrial ATPase enzymes of single gene, nuclear mutants of S. cerevisiae are unaffected if a single cytochrome is missing, however if the mutation causes a deficiency in two or more cytochromes then the ATPase activity of the mitochondria has "petite" characteristics (Subik et al, 1970; Subik et al, 1972).

Nuclear or cytoplasmic mutants of S. cerevisiae which are deficient in cytochrome content or in ATPase activity have made some contribution to the study of oxidative phosphorylation. Observations on the biogenesis of mitochondria have allowed some resolution of the system into its component parts (Mason et al, 1972; Tzagoloff et al, 1973). Characterisation of mutants of S. cerevisiae resistant to specific inhibitors of oxidative phosphorylation should also prove useful. If the mutation is cytoplasmic and the resistance phenomenon occurs at the mitochondrial level then there is the possibility of showing that a mutation in mitochondrial DNA results in a change in a single mitochondrially synthesised protein. This change may have resulted in specific effects on oxidative phosphorylation. The existence of uncoupler binding sites, coupled with resistance to uncouplers at the mitochondrial level, raises the possibility of correlating changes at these sites with concomitant deficiencies in oxidative phosphorylation in these mutants.

CHAPTER 4. Characteristics of Cells and Mitochondria of Saccharomycopsis lipolytica grown on various substrates including n-alkanes.

INTRODUCTION

One possible result of mutation to uncoupler resistance, is an increase in the efficiency of oxidative phosphorylation. This would lead to a higher growth yield and possibly a faster growth rate than the wild type.

British Petroleum are at present growing species of Saccharomycopsis yeast on a large scale for animal feed. These organisms are grown in continuous culture utilising long chain aliphatic hydrocarbons as sole carbon scource (Wilkinson, 1971; Klug and Markovetz, 1971). Any increase in the growth yield of these yeast would contribute to the efficiency and profitability of this operation. Although none of the uncoupler resistant mutants of S. cerevisiae chosen for study in previous Chapters have improved growth characteristics over the wild type; the properties of Saccharomycopsis lipolytica cells grown on various carbon sources, including n-alkanes, were examined.

The problems entailed in growing yeast on hydrocarbon substrates have recently come under investigation. Since the carbon scource is immiscible with water an important consideration has been the mechanism of uptake of the n-alkane. Both direct uptake of hydrocarbon droplets (Aiba et al, 1969a, b) and utilisation in the dissolved state (Goma et al, 1973) have been proposed. The degradation of hydrocarbons by microorganisms has been discussed by Klug and Markovetz, (1971) and the break down of n-alkanes by the yeast Candida tropicalis has been described in detail (Gallo et al, 1973a; Gallo et al, 1973b). The hydrocarbon is thought to be oxidised to a primary alcohol, then through the aldehyde to the fatty acid. It is presumed that it is then metabolised through β-oxidation.

There are variations in the characteristics of growth of S. lipolytica between different substrates and these may be correlated with changes in the cytochrome contents of the cells. In order to provide some information on the oxidative phosphorylation process the mitochondrial cytochrome profiles and Mg^{2+}-dependent, ATPase activities were also measured. Some of the properties of these S. lipolytica mitochondria are in great contrast to those of S. cerevisiae (Houghton et al, 1973; Skipton et al, 1973).

METHODS AND MATERIALS

Description of Yeast Strain.

Strains of Candida lipolytica are generally found in the haploid state and, being heterothallic, they do not sporulate when grown individually on appropriate media. However, if strains of opposite type are mated then ascospores may be produced. Diploids have also been isolated but they are not normally ascosporogenous. However, C. lipolytica, strain NRRL YB-423 is such a strain and produces one to four spores per ascus (Wickerham et al, 1970). The germinability of the spores is very low (0.1%) but several asexually reproducing clones were obtained eg. YB-423-3 (mating type A) and YB-423-12 (mating type B). If YB-423-12 is mated with another haploid strain, YB-421, then the ascospores produced have a much higher viability (12%). These observations indicate that hybridisation studies are possible in order to obtain diploid cells with more desirable growth characteristics. This could lead to improvements in the efficiency of hydrocarbon fermentation processes. It is possible to increase the fertility of the heterothallic segregant strains, obtained from YB-423, by suitable choice of growth medium (Herman, 1971).

Wickerham et al, (1969) have concluded that this organism should be described as Endomycopsis lipolytica in the absence of any publication that describes Candida lipolytica as a perfect species. However, von Arx, (1972) has proposed that the generic name Endomycopsis is incorrect and should be discarded in favour of the genus Saccharomycopsis. The strain of yeast used in this Chapter will therefore be referred to as Saccharomycopsis lipolytica, strain YB-423; it produces true hyphae as well as budding cells, and has an ascosporogenous, heterothallic sexual cycle.

Bassel et al, (1971) introduced genetic markers into haploid strains and have shown hybrid formation with complementation and meiotic recombination in individual ascospores. Bassel and Mortimer, (1973) find such hybrids of S. lipolytica with a spore viability of 70-80% allowing recovery of many intact

tetrads. This has allowed genetic analysis of n-alkane utilisation, which is shown to proceed through a fatty acid intermediate as proposed by other workers (Klug and Markovetz, 1971).

Samples of S. lipolytica, strains YB-423, YB-423-3 and YB-423-12, were obtained from B.P. Research (Chertsey Road, Sunbury-on-Thames, Middx.). They were maintained at 5°C on plates (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar) and subcultured every month.

Growth of S. lipolytica.

S. lipolytica was grown in 10 ltr. cultures in a fermenter (New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A.) in the dark at 30°C in a medium containing per litre of distilled water:- 0.5% (w/v) yeast extract; 2.5 g NH_4Cl; 7.0 g KH_2PO_4; 3.0 g $Na_2HPO_4.2H_2O$; 0.2 g $MgSO_4.7H_2O$; 0.1 g NaCl; 0.5 mg HBO_3; 0.4 mg $CuSO_4.5H_2O$; 0.1 mg KI; 0.4 mg $MnSO_4$; 0.2 mg $Na_2MoO_4.2H_2O$; 0.4 mg $ZnSO_4.7H_2O$; 0.2 mg $FeCl_3$. 0.1 g $CaCl_2.2H_2O$ was added after autoclaving. Ethanol (0.5 - 1.0% v/v), glucose (0.6% - 10.0% w/v) or n-alkane (0.1 - 0.4% v/v) were used as carbon scources as indicated in the text. Tributyl citrate at a concentration of 0.1 - 0.2 ml/ltr was used as antifoaming agent and the pH of the medium was between 5.8 and 6.0. Stirring speed was 500 revs per min and compressed air was passed through the culture at 1 ltr air/min/ltr medium. A 1.0% (v/v) inoculum from a starter culture grown for 24 hr on 0.5% (v/v) ethanol was used in every case.

Growth and Respiratory Activity of S. lipolytica on Ethanol.

Cells were grown in 500 ml medium in 2 ltr conical flasks, baffled for efficient aeration. The medium was as above but without the antifoaming agent, ethanol was at a concentration of 0.5% (v/v). The inoculum was a 1.0% (v/v) sample of a starter culture grown for 24 hr under the same conditions. The flasks were incubated at 30°C in the dark and agitated at 200-250 revs per min on a Mark 5 shaker, (L. H. Engineering Co., Stoke Poges, Bucks, U.K.). Samples were periodically withdrawnfor determination of the concentration of the cells by dry weight. At the same time, an aliquot of the culture was centrifuged and the cells washed, and resuspended in 50 mM potassium phosphate, pH 6.0. The respiratory capacity of the cells on ethanol was measured using

a Rank oxygen electrode, as described for S. cerevisiae (Methods, Chapter 2) and using phosphate buffer, pH 6.0 instead of potassium phthalate.

Preparation of Mitochondria (Braun shaker).

These were normally prepared from fermenter grown cells, grown to early stationary phase on any carbon scource (details in text). This procedure is described in Chapter 2 for S. cerevisiae and exactly the same methods were used here. These mitochondria were gradient purified before use in assays.

Preparation of Mitochondria (Snail enzyme).

S. lipolytica cells grown in the fermenter to log phase on ethanol (1.0% v/v) were used. This preparation is the same as described in Chapter 3 for S. cerevisiae.

Cytochrome Contents of S. lipolytica.

The cytochrome contents of S. lipolytica cells were determined by reduced minus oxidised difference spectroscopy as described for S. cerevisiae cells. (Chapter 2). These measurements were done at room temperature using a Unicam SP 1800 scanning sprectrophotometer with a turbid suspension facility. Reduced minus oxidised cytochrome spectra of S. lipolytica mitochondria were also obtained in a similar fashion.

Mitochondrial ATPase Assay.

The reaction medium contained, in a final volume of 1 ml, 5 mMATP, 2 mM $MgCl_2$, 50 mM Tris-maleate, pH 6.0-7.5, or 50 mM Tris-HCl, pH 7.5-10.0; 100-200 μg of mitochondrial protein was used for each assay. The temperature of the assay was 30°C and 10 min prein cubations were done with DNP or oligomycin before starting by addition of ATP. DNP ordigomycin were added from concentrated solutions in methanol. The assays were continued for 10 min before termination by the addition of 1 ml of 10% (w/v) trichloroacetic acid. After centrifugation, 0.5 ml samples were used for the determination of phosphate by the method of King, (1932).

Dry Weight and Protein Estimations.

Dry weights of intact cells were measured by filtration onto Whatman GF/C glass fibre paper (2.4 cm diameter) and dried in an oven at 110°C to constant weight for 2-3 days. Protein estimations were by the Lowry procedure (Lowry et al, 1951).

Materials.

Yeast extract was obtained from Difco (Difco Laboratories, Detroit, Michigan, U.S.A.) and peptone and agar from Oxoid (Oxoid Ltd., London.). ATP and oligomycin were bought from Sigma (Sigma (London) Chemical Co., Kingston-on-Thames). DNP was obtained from BDH (B.D.H. Chemicals Ltd., Poole, Dorset). All other chemicals were of Analar grade where necessary.

RESULTS

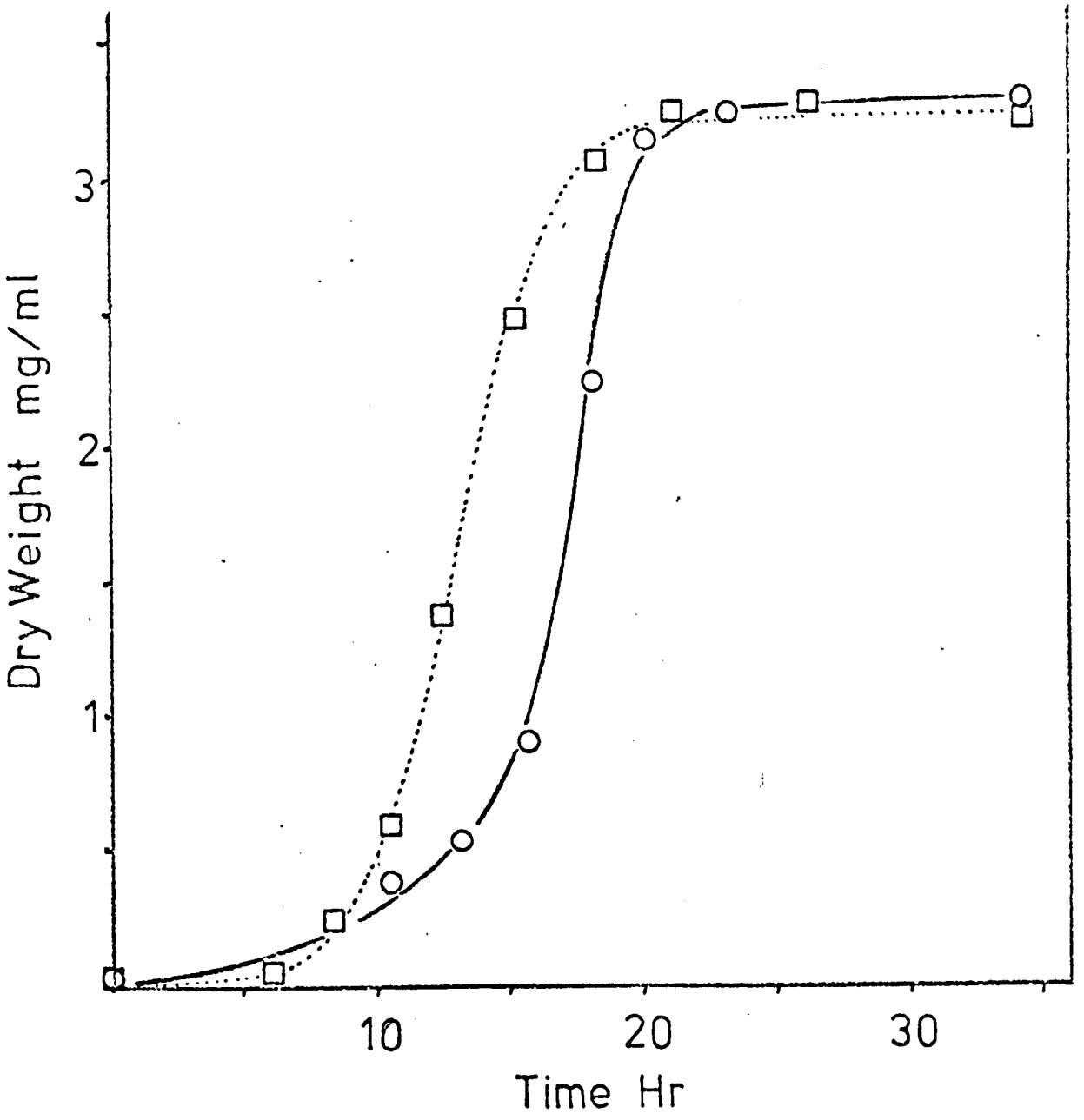
Growth Characteristics.

The growth of S. lipolytica, strain YB-423, on ethanol (Figure 4. 1) is comparable with that of S. cerevisae, strain D22, (Figures 2. 1 and 2. 4). Although there were differences in the makeup of the culture media, the maximal growth rates areapproximately the same. In both cases, the amount of cells produced at entry into stationary phase was in the same range (3. 5-4. 0 mg dry weight per ml). S. lipolytica cannot be grown fermentatively on glucose (Wickerham et al, 1969) but a growth curve under aerobic conditions is shown in Figure 4. 1. This may be contrasted with the characteristics of S. cerevisiae, strain D22, grown aerobically on glucose (Figure 2. 8). The lag phase for S. cerevisiae was virtually non-existent whereas for S. lipolytica it lasted for about 6 hr. Second, growth of S. lipolytica in the initial stages of log phase was much more rapid than that of S. cerevisiae. Both however approached the ethanol supported growth rate in middle to late log phase. Owing to differences in glucose concentration; 0. 4% (w/v) for S. cerevisiae and 0. 6% (w/v) for S. lipolytica; the growth yields are not the same, S. lipolytica being the higher at 3. 5 mg dry weight per ml.

The rate of respiration of S. lipolyticathroughout batch culture on ethanol (0. 5% v/v) was measured (Figure 4. 2). The oxygen uptake by S. lipolytica cells was much higher than that achieved by S. cerevisiae (Figure 2. 4). In both cases the maximal rate of respiration was attained at approximately halfway through the logarithmic phase of growth (Singh et al, 1972). However, that of S. cerevisiae was 84 nmoles 0_2 per min per mg dry weight but that of S. lipolytica was 220 nmoles 0_2 per min per mg dry weight. Both decreased to lower values (40-50 nmoles 0_2 per min per mg dry weight) on reaching stationary phase.

The growth of S. lipolytica in batch culture on n-tetradecane (0. 1% v/v) is also shown in Figure 4. 2. S. lipolytica, strain YB-423, can utilise hydrocarbons as substrate and is able to grow in liquid culture on n-alkanes of 10-20 carbon atoms in chain length. All studies described here were done using fermenter grown, batch cultures. For n-tetradecane at 0. 1% (v/v) (Figures 4. 2 and 4. 3) the lag phase was 18 hr, in contrast to 10 hr for the ethanol grown cells under

Figure 4.1



Growth curve of S. lipolytica. The cells were grown in the fermenter.

O - O 0.5% (v/v) ethanol.

□---□ 0.6% (w/v) glucose.

FIGURE 4.2

Growth curves and oxygen uptake of S. lipolytica. Shake flask culture on 0.5% (v/v) ethanol:-

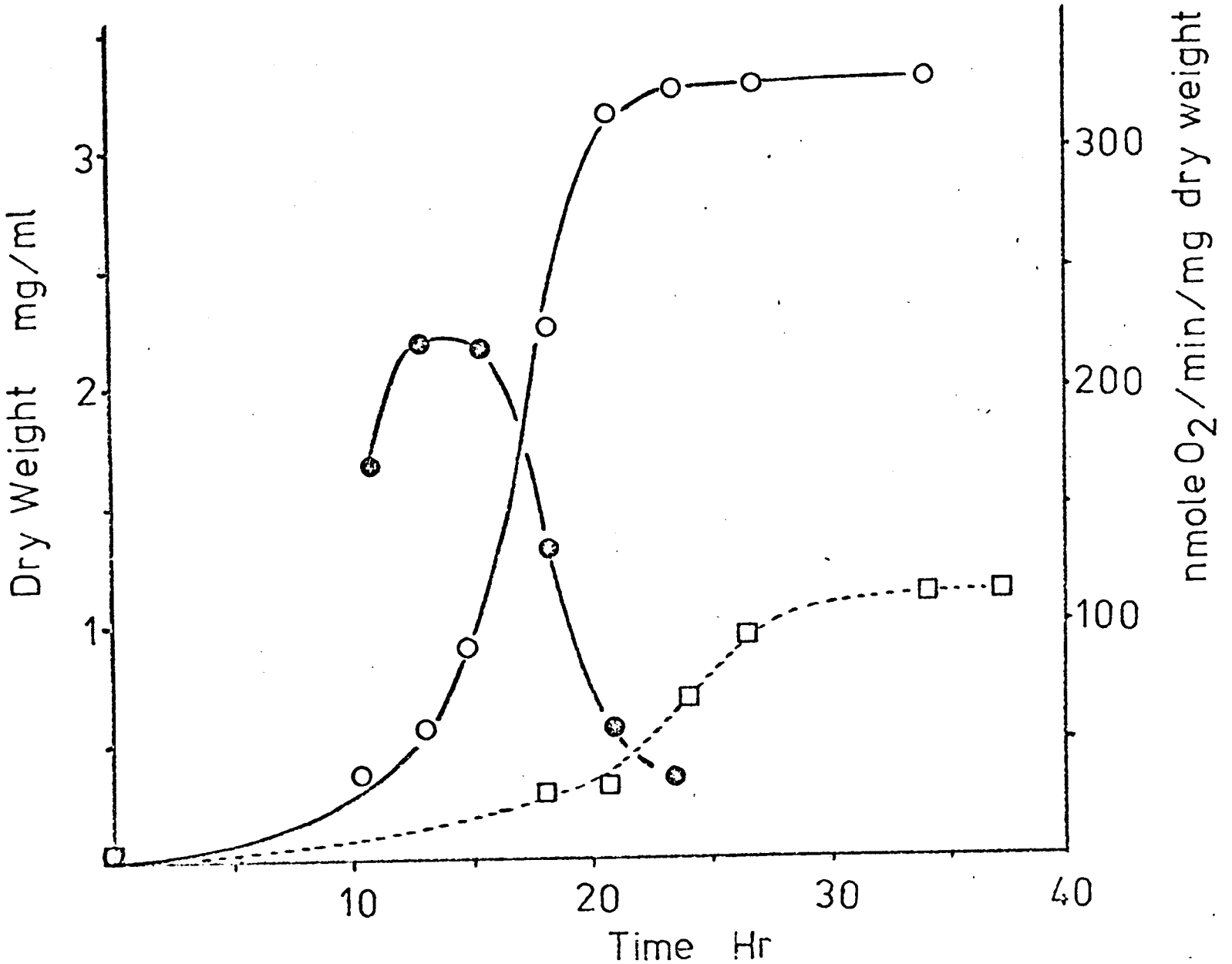
O - O Dry weight mg/ml.

● - ● nmole 0_2/min/mg dry weight.

Fermenter grown culture on 0.1% (v/v) n-tetradecane:-

□-----□ Dry weight mg/ml.

Figure 4.2


Dry Weight mg/ml
3
2
1
300
200
100
nmole O_2/min/mg dry weight
10
20
30
40
Time Hr

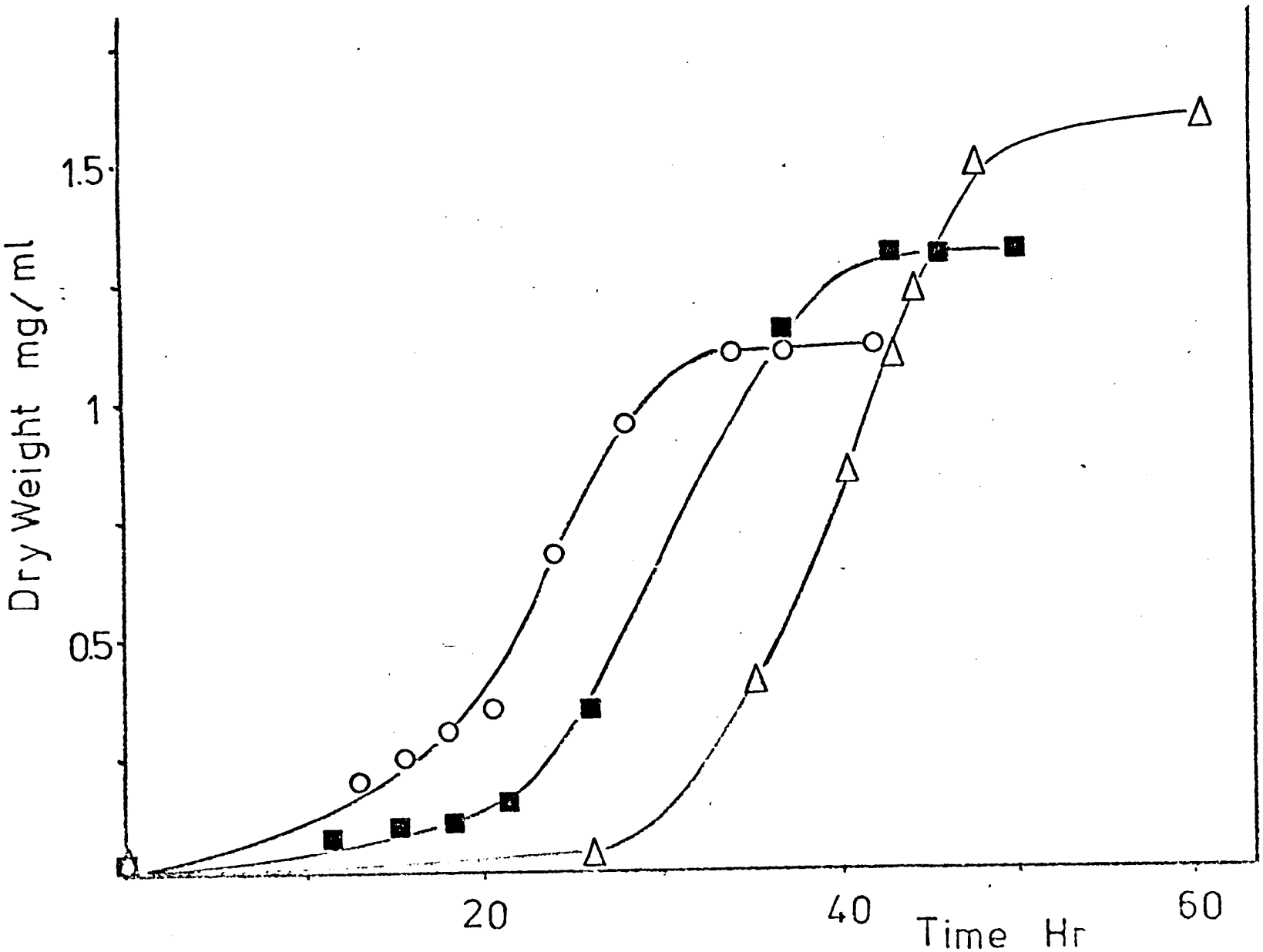


Figure 4.3

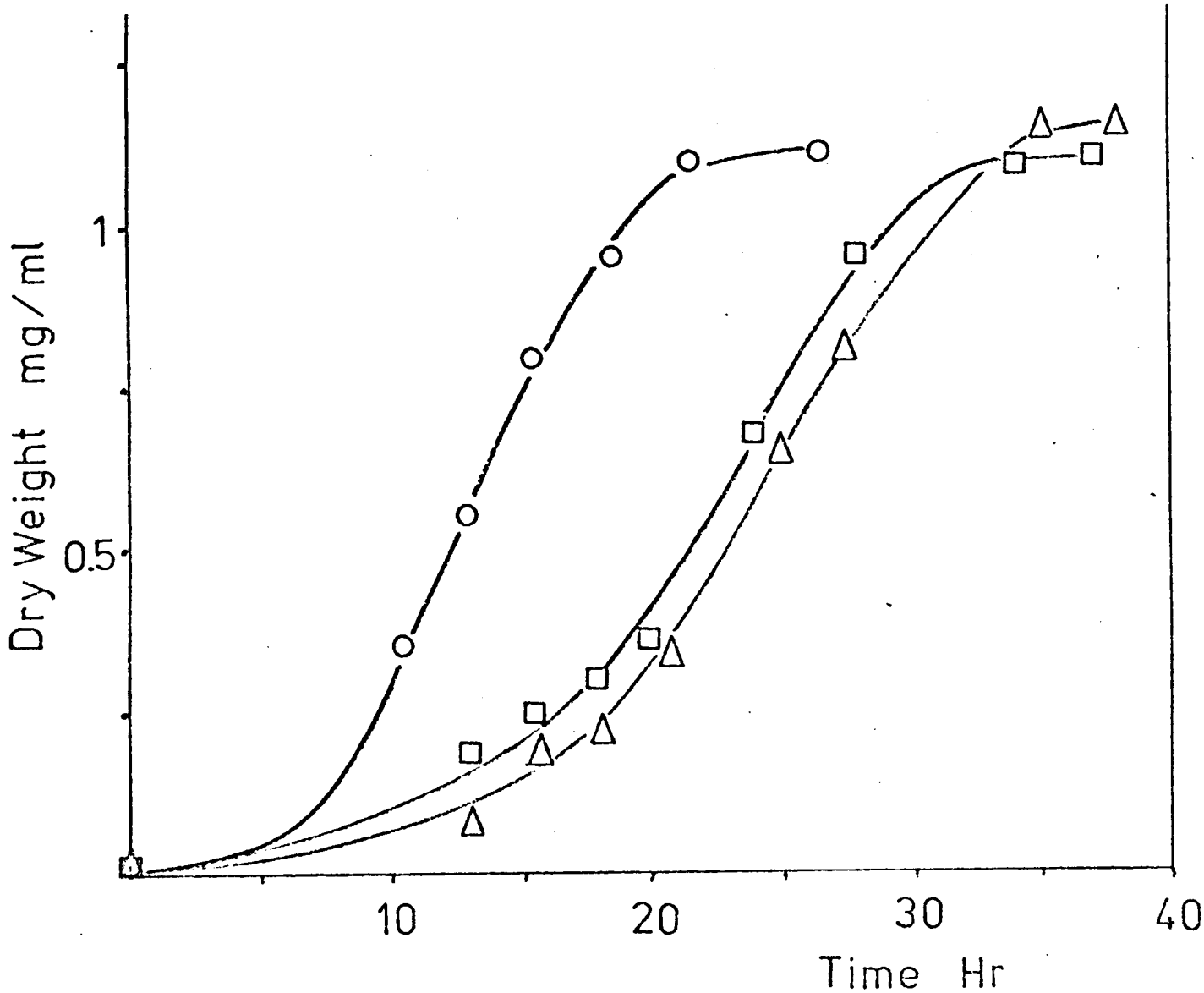
Growth curves of S. lipolytica on different concentrations of n-tetradecane in the fermenter.

O - O 0.1% (v/v).

■ - ■ 0.2% (v/v).

Δ - Δ 0.4% (v/v).

Figure 4.4



Growth curves of S. lipolytica on different n-alkanes in the fermenter.

O - O n-decane 0.1% (v/v).

□ - □ n-tetradecane 0.1% (v/v).

Δ - Δ n-hexadecane 0.1% (v/v).

the same conditions (Figure 4.1). It should also be noted that the yield of cells (1.1 mg dry weight per ml after 34 hr) is small, considering the relatively high carbon content of the substrate, and especially so when compared to growth on ethanol. Experiments to measure rates of oxygen uptake were unsuccessful owing to the insolubility of the hydrocarbons and the tendency of the yeast cells to adhere to the paraffin droplets in the culture.

Most of the studies on the growth of yeast on n-alkanes have been done using Candida species. Differences in cell yield on varying chain length do not follow any general rule (Klug and Markovetz, 1971). Growth of S. lipolytica on increasing concentrations of n-tetradecane (Figure 4.3) shows no corresponding increase in cell yield. These results may be contrasted with cells grown on ethanol where there is an approximate doubling in cell yield in going from 0.5% (v/v) to 1.0% (v/v) ethanol (results not shown). When the concentration of n-tetradecane was increased four-fold there was a less than two-fold increase in the cell yield. As the n-alkane concentration was increased, the length of the lag phase was extended (from 18 hr to 25 hr) even though all other external growth conditions remained the same. This again is in contrast to observations on ethanol grown cells where no change in this region of the growth curve is seen on alteration of the substrate concentration.

There is no corresponding increase in the cell yield in going from growth on n-decane to n-tetradecane or to n-hexadecane at the same concentration (Figure 4.4) even though there is an increase in the carbon content of the substrate. It is evident however, that the lag phase is again affected. On n-decane (6 hr) this is much shorter than the corresponding phase on n-tetradecane or on n-hexadecane (18 hr). However the generation times on all the alkanes tested at whatever concentration, were the same, at about 5.5 hr (Figures 4.3 and 4.4).

Cytochrome Contents of Cells and Mitochondria.

The cytochrome difference spectra of S. lipolytica, strain YB-423 cells at early stationary phase after growth on 0.5% (v/v) ethanol are illustrated in Figure 4.5. These may be contrasted with the difference spectra of S. cerevisiae, strain D22 cells grown on ethanol in a shake flask (Figure 2.21) or fermenter (Figure 2.18). S. lipolytica is characterised by a large cytochrome $a a_3$ absorption at 605 nm and a relatively broad band (560-565 nm) due to b-type cytochromes.

FIGURE 4.5

Reduced minus oxidised cytochromedifference spectra of S. lipolytica cells grown to stationary phase on 0.5% (v/v) ethanol.

1. Shake flask culture (25 mg dry weight/ml).
2. Fermenter grown culture (15.2 mg dry weight/ml).

FIGURE 4.6

Reduced minus oxidised cytochrome difference spectra of S. cerevisiae, Strain D22, and S. lipolytica cells grown aerobically in shake flask culture on glucose (5% w/v) and harvested in log phase.

(----------) S. cerevisiae 26 mg dry weight/ml.

(——————) S. lipolytica 9 mg dry weight/ml.

Figure 4.5 A

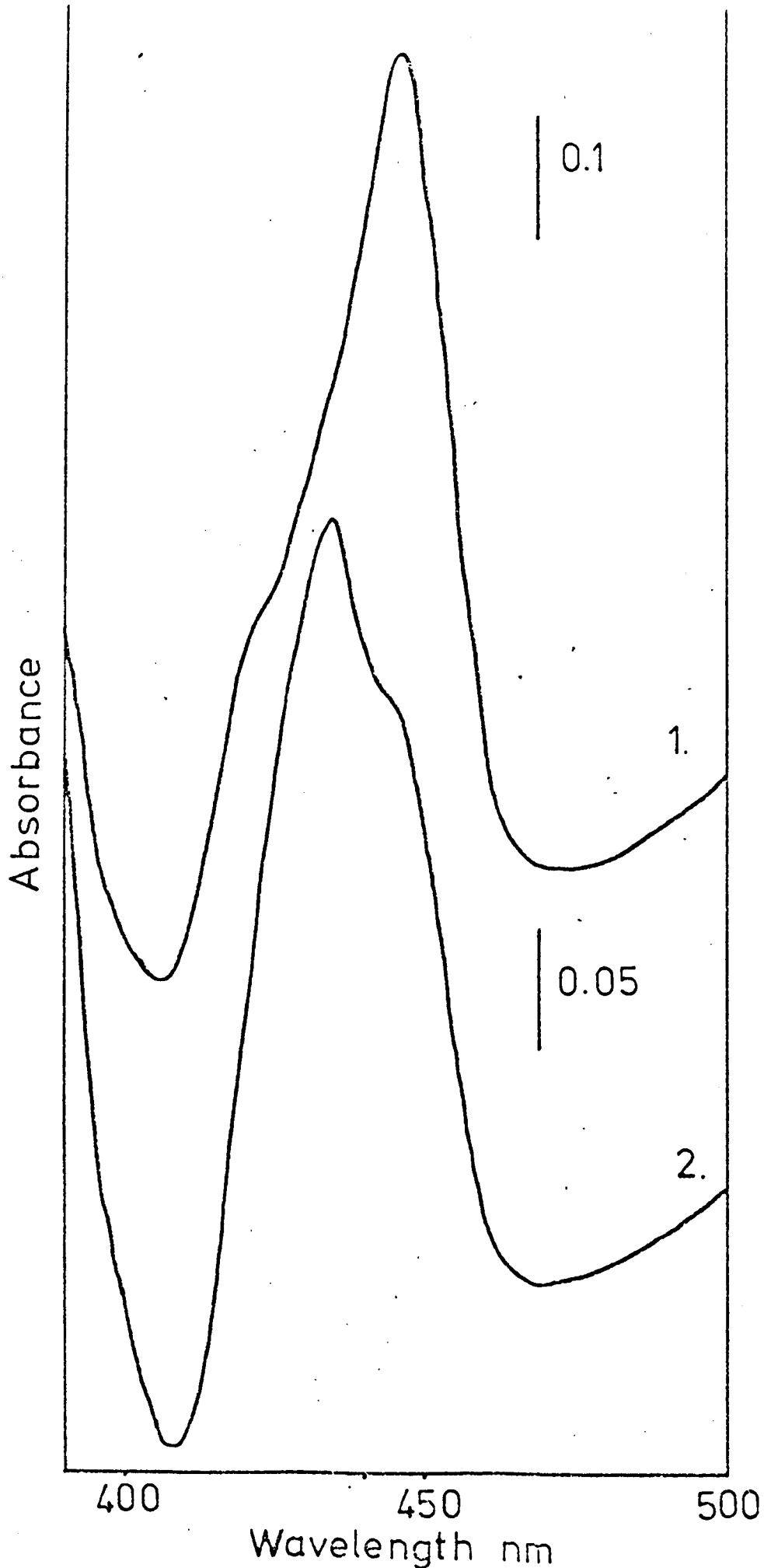


Figure 4.5 B

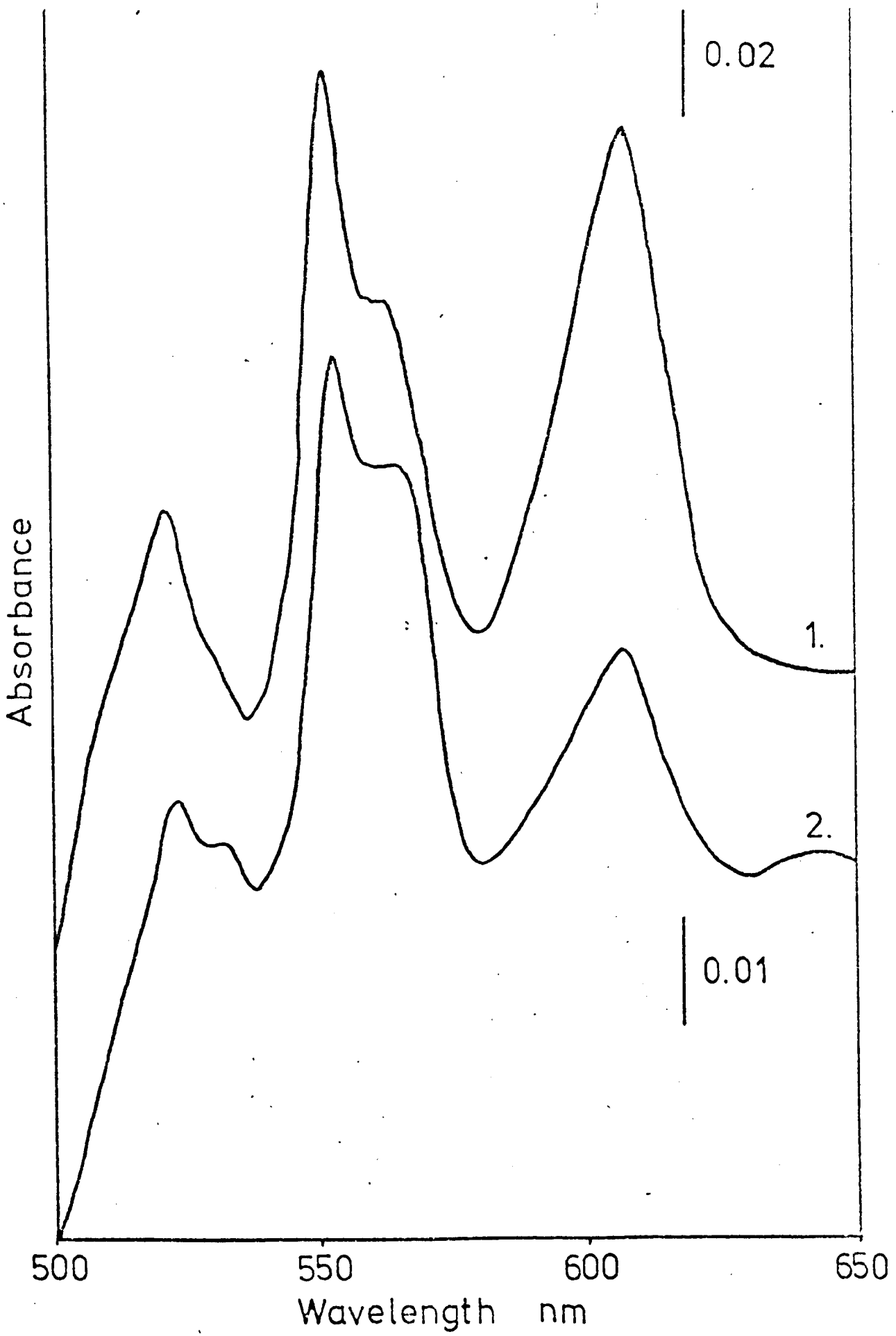


Figure 4.6 A

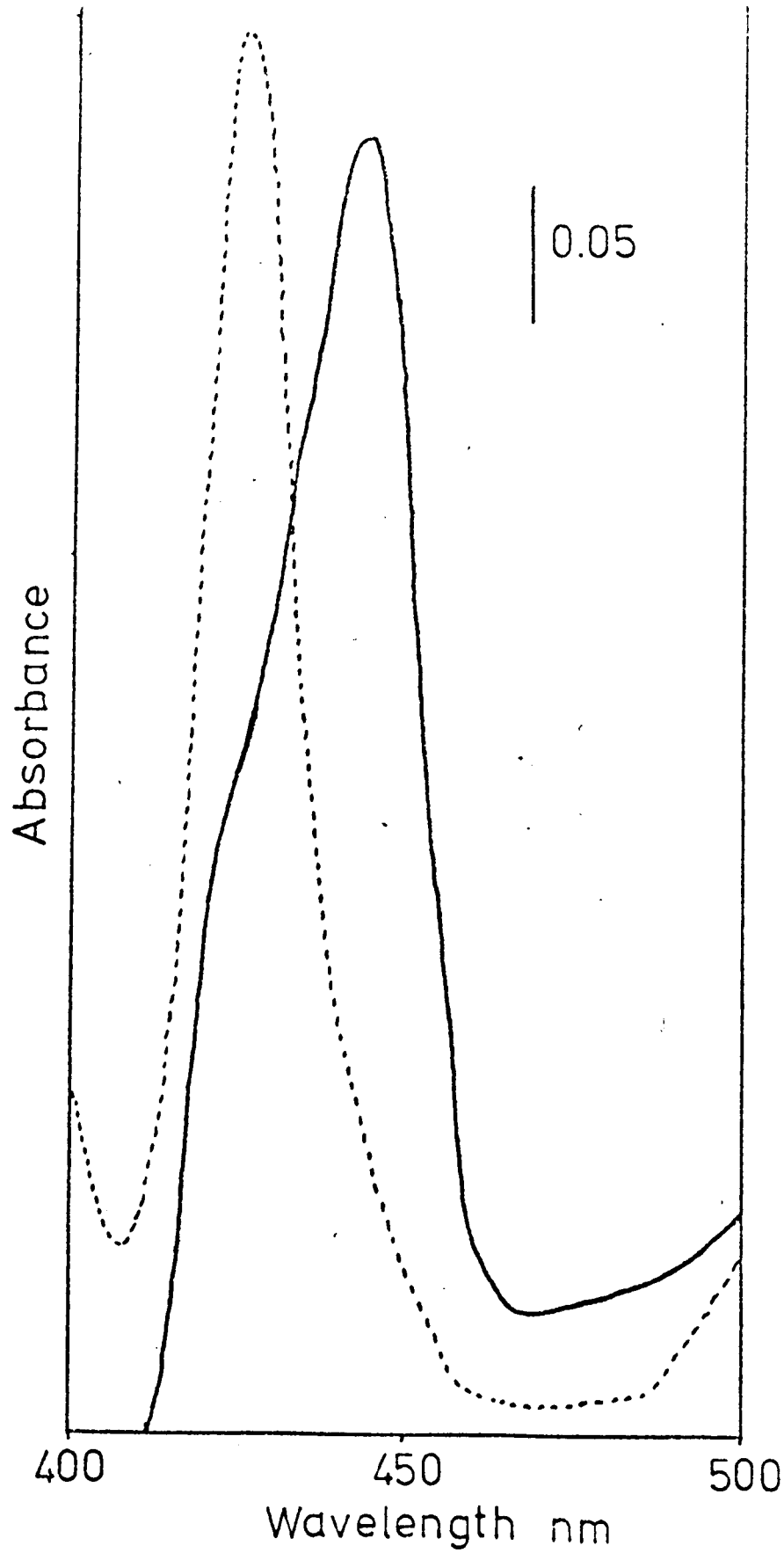


Figure 4.6 B

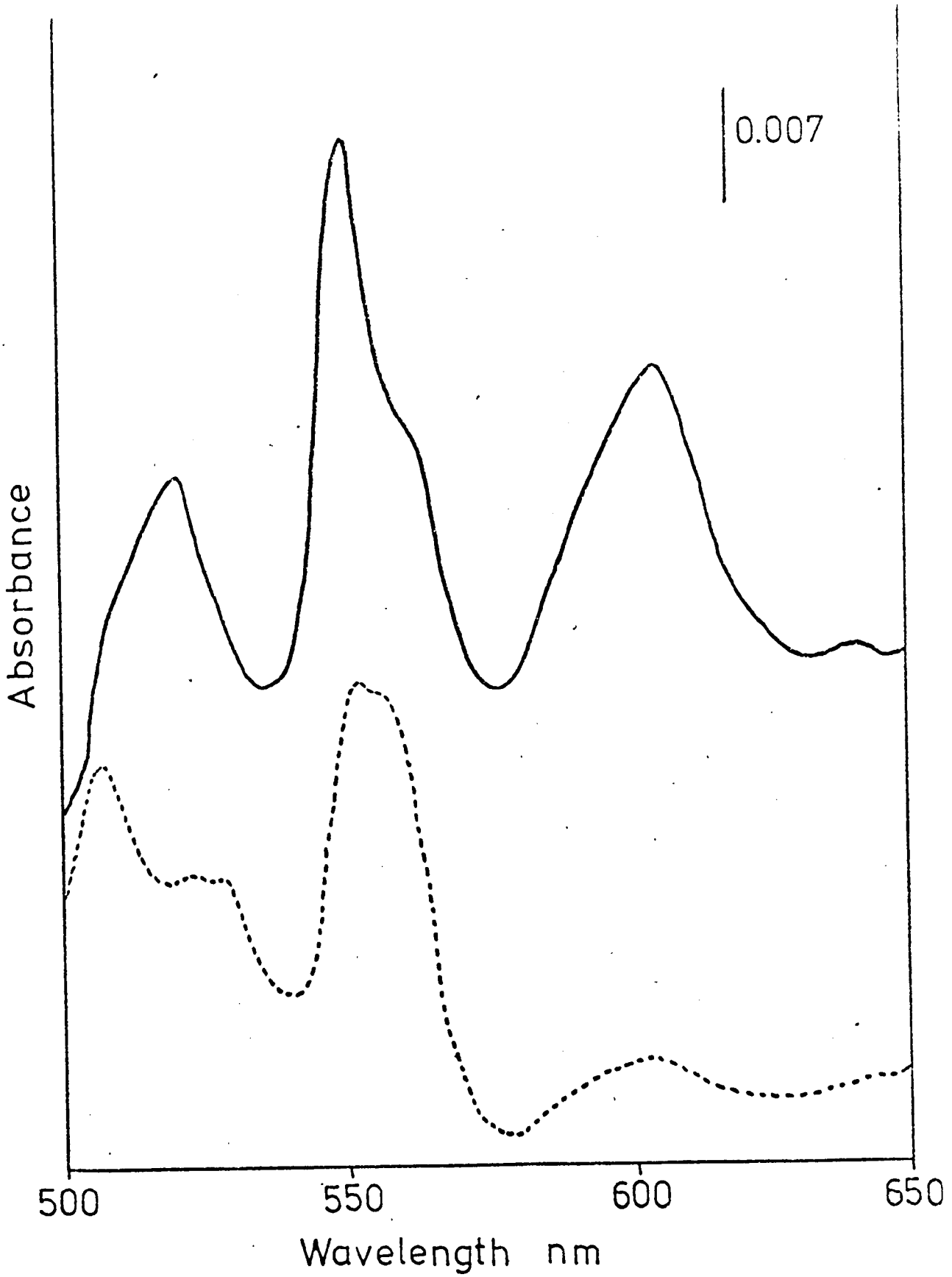


FIGURE 4.7

Reduced minus oxidised cytochrome difference spectra of S. lipolytica cells grown in the fermenter on n-tetradecane (0.2% v/v).

Stat.	Stationary phase	(28.5 mg dry weight/ml)
Log.	Logarithmic phase	(20.5 mg dry weight/ml)

FIGURE 4.8

Reduced minus oxidised cytochrome difference spectra of S. lipolytica cells grown in the fermenter to stationary phase on n-pentadecane (0.2% v/v). Cell concentration is 26 mg dry weight/ml.

Figure 4.7 A

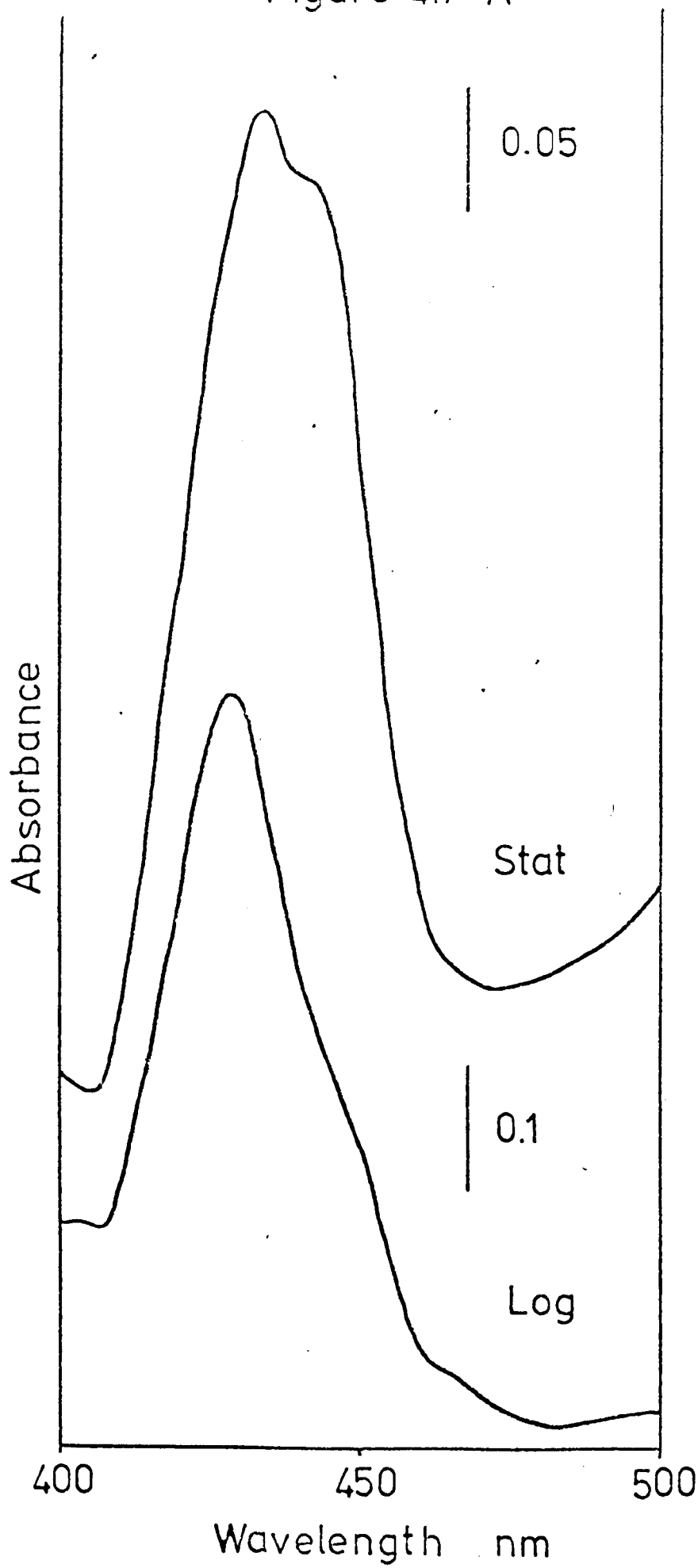


Figure 4.7 B

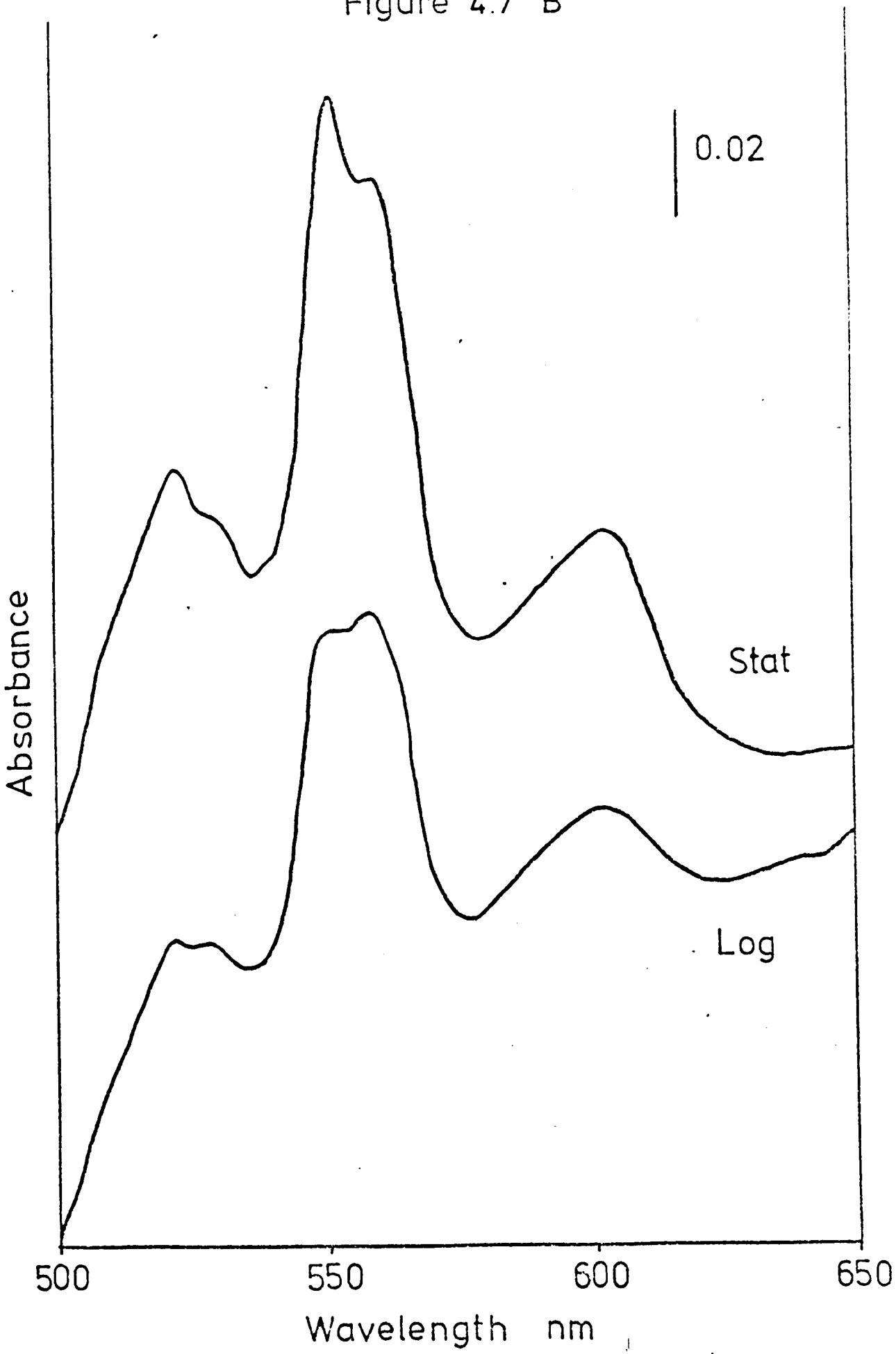


Figure 4.8

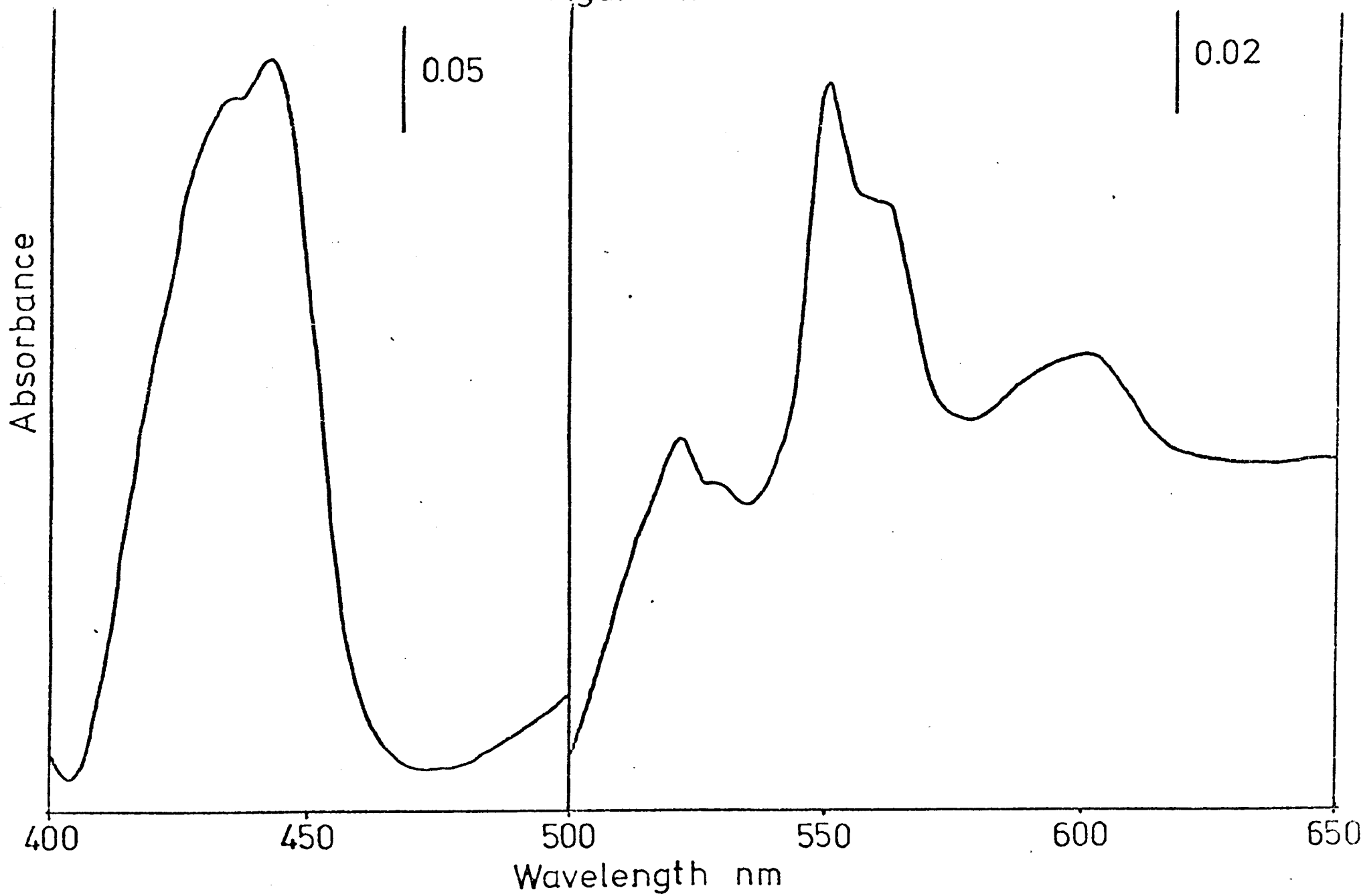


TABLE 4.1.

Cytochrome contents of S. lipolytica and S. cerevisiae grown on different substrates.

	Substrate	Growth Conditions	Growth Phase	nmole cytochrome/mg dry weight			Relative Percentage		
				c	b	aa_3	$\frac{c}{c}$	$\frac{b}{c}$	$\frac{aa_3}{c}$
	S. lipolytica								
1.	Ethanol	Shake Flask	Stationary	0.23	0.124	0.168	100	54	73
2.	Ethanol	Fermenter	Stationary	0.18	0.083	0.089	100	46	49
3.	Glucose	Shake Flask	Logarithmic	0.18	0.074	0.084	100	41	46
4.	n-Tetradecane	Fermenter	Logarithmic	0.149	0.135	0.028	100	91	19
5.	n-Tetradecane	Fermenter	Stationary	0.15	0.097	0.059	100	61	37
6.	n-Pentadecane	Fermenter	Stationary	0.15	0.091	0.035	100	61	23
	S. cerevisiae (strain D22)								
7.	Ethanol	Shake Flask	Stationary	0.186	0.061	0.046	100	33	25
8.	Ethanol	Fermenter	Stationary	0.168	0.05	0.019	100	30	11
9.	Glucose	Shake Flask	Logarithmic	0.04	0.026	0.0039	100	65	10

Notes

1. Results 1.-6. in this Table are analyses of Figures 4.5-4.8 respectively.
2. Results 7. and 9. in this Table are analyses of Figures 2.21 and 4.6 respectively.

Differences between S. lipolytica and S. cerevisiae cells are further emphasised by examination of the absorption bands in the Soret region. In the case of flask grown S. lipolytica there was a large cytochrome aa_3 peak (445 nm) with shoulders at 430 nm(cytochrome b) and 415 nm (cytochrome c). In contrast to Saccharomyces sp. there is a large amount of cytochrome aa_3, relative to cytochrome c. The growth conditions affect the cytochrome contents of S. lipolytica. Fermenter grown cultures contain less cytochrome aa_3 than flask grown cells.

The difference spectra of S. cerevisiae and S. lipolytica cells grown aerobically on glucose (5% w/v) in shake flasks and harvested in log phase are shown in Figure 4.6. The S. cerevisiae cytochromes are typically glucose repressed (Jayaraman et al, 1966) while the S. lipolytica spectrum is not. Although there are less cytochromes in glucose grown compared with ethanol grown Saccharomycopsis cells, this yeast is much less subject to glucose repression than S. cerevisiae. The cytochrome content of S. lipolytica grown to log phase on glucose is comparable with fully derepressed S. cerevisiae cells grown on ethanol (Table 4.1). The cytochrome aa_3 content of glucose and of ethanol grown S. lipolytica cells was two and four fold, respectively, higher than that of ethanol grown S. cerevisiae.

Different cytochrome profiles were observed when S. lipolytica cells were grown on n-alkanes. Figure 4.7 illustrates the cytochrome spectra obtained from n-tetradecane, grown cells harvested in log and stationary phases. These show noticeable changes in going from a "repressed" (log phase) to a "derepressed" (stationary phase) state. This is reminiscent of glucose derepression in S. cerevisiae (Slonimski, 1953; Jayaraman et al, 1966). The variations in the spectra in the Soret region were particularly dramatic. In the case of log phase cells the cytochrome aa_3 absorption appeared as a small shoulder on the large cytochrome b peak, while in stationary phase the cytochrome aa_3 maximum was almost equal to cytochrome b. The difference spectrum of stationary phase cells grown on n-pentadecane in a fermenter (Figure 4.8) is similar to that of the n-tetradecane grown cells except that there is less cytochrome aa_3 (Table 4.1). Cells grown on n-alkane always have less cytochrome aa_3 than ethanol grown cells (Table 4.1).

Mitochondria were prepared from fermenter grown cultures of S. lipolytica by the Braun shaker method. Again quantitative estimation of the cytochrome

TABLE 4.2.

Cytochrome contents of S. lipolytica and S. cerevisiae mitochondria from cells grown on different substrates.

	Substrate	Growth Conditions	Mitochondrial Preparation	nmole cytochrome/mg protein			Relative Percentage		
				c	b	aa_3	$\frac{c}{c}$	$\frac{b}{c}$	$\frac{aa_3}{c}$
	S. lipolytica								
1.	Ethanol	Fermenter	Braun Shaker	1.36	0.84	0.74	100	62	54
2.	n-Tetradecane	Fermenter	Braun Shaker	0.565	0.445	0.245	100	79	43
	S. cerevisiae (strain D22)								
3.	Ethanol	Shake Flask	Snail Enzyme	1.09	0.59	0.22	100	54	24
4.	Ethanol	Fermenter	Braun Shaker	1.06	0.55	0.112	100	52	11

Notes.

1. Cells used for the Braun shaker preparation were grown to stationary phase, those for snail enzyme treatment were harvested at log phase.
2. Results for S. cerevisiae, strain D22 are obtained from Table 2.12.

contents were complicated by variable losses during the isolation procedure. It is evident however that mitochondria from S. lipolytica cells, grown on ethanol, have a much higher overall cytochrome content than the corresponding mitochondria from S. cerevisiae (Table 4. 2). In particular, the cytochrome b and $a a_3$ contents of Saccharomycopsis mitochondria are 1. 5 and at least 3. 5 times, respectively, higher than in S. cerevisiae. The amounts of cytochromes b and $a a_3$ in mitochondria from n-tetradecane grown cells of S. lipolytica were much less than in the ethanol grown case (Table 4. 2).

Consideration of the relative amounts of cytochromes in S. cerevisiae and S. lipolytica emphasises the differences between these yeasts (Tables 4. 1 and 4. 2). These data clearly show that S. lipolytica cells are comparatively rich in cytochromes b , and $a a_3$ with S. cerevisiae cells rich in cytochrome c. The corresponding ratios for cells grown on glucose indicate that there is some repression of cytochromes b and $a a_3$ in S. lipolytica.

S. lipolytica grown on n-tetradecane showed changes in cytochrome ratios dependent on the phase of growth. Log phase cells have relatively high levels of cytochrome b while in stationary phase cells there is an increase in the amount of cytochrome $a a_3$. Whatever the growth phase the n-alkane grown cells were always characterised by higher levels of b type cytochromes than the ethanol grown cells (Table 4. 1). This is also reflected in the results for n-pentadecane grown cells.

Cytochrome ratios in isolated mitochondria confirm the relatively high cytochrome b and $a a_3$ contents of S. lipolytica compared to S. cerevisiae. This was the case for mitochondria isolated from both ethanol and n-tetradecane grown cells of S. lipolytica (Table 4. 2). Mitochondria from n.-alkane grown cells have a higher relative content of cytochrome/b than those from S. lipolytica cells grown on ethanol. This is in accord with the data in Table 4. 1 and is due to a lowering in the level of cytochrome c, together with an increase in the amount of cytochrome b.

Mitochondrial ATPase.

Two maxima, at pH 7. 0 and pH 8. 5, were observed in the pH profile of the mitochondrial ATPase activity of S. lipolytica strain YB-423 cells grown to early stationary phase on ethanol. These maxima were more clearly resolved in the case of mitochondria isolated from protoplast lysates (Figure 4. 9). In contrast to S. cerevisiae (Figure 3. 21) the specific activity

FIGURE 4.9

The effect of pH on the mitochondrial ATPase in S. lipolytica cells grown in the fermenter on different substrates. For preparation of Braun shaker mitochondria the cells were harvested in stationary phase, and for snail enzyme mitochondria in log phase. All mitochondria were gradient purified before use.

□ - □ n-tetradecane (0.2% v/v); Braun shaker mitochondria.

O - O ethanol (0.5% v/v); Braun shaker mitochondria.

▲ - ▲ ethanol (1.0% v/v); snail enzyme mitochondria.

Δ - Δ ethanol (1.0%v/v); snail enzyme mitochondria + 0.1 mM DNP , in assay.

Figure 4.9

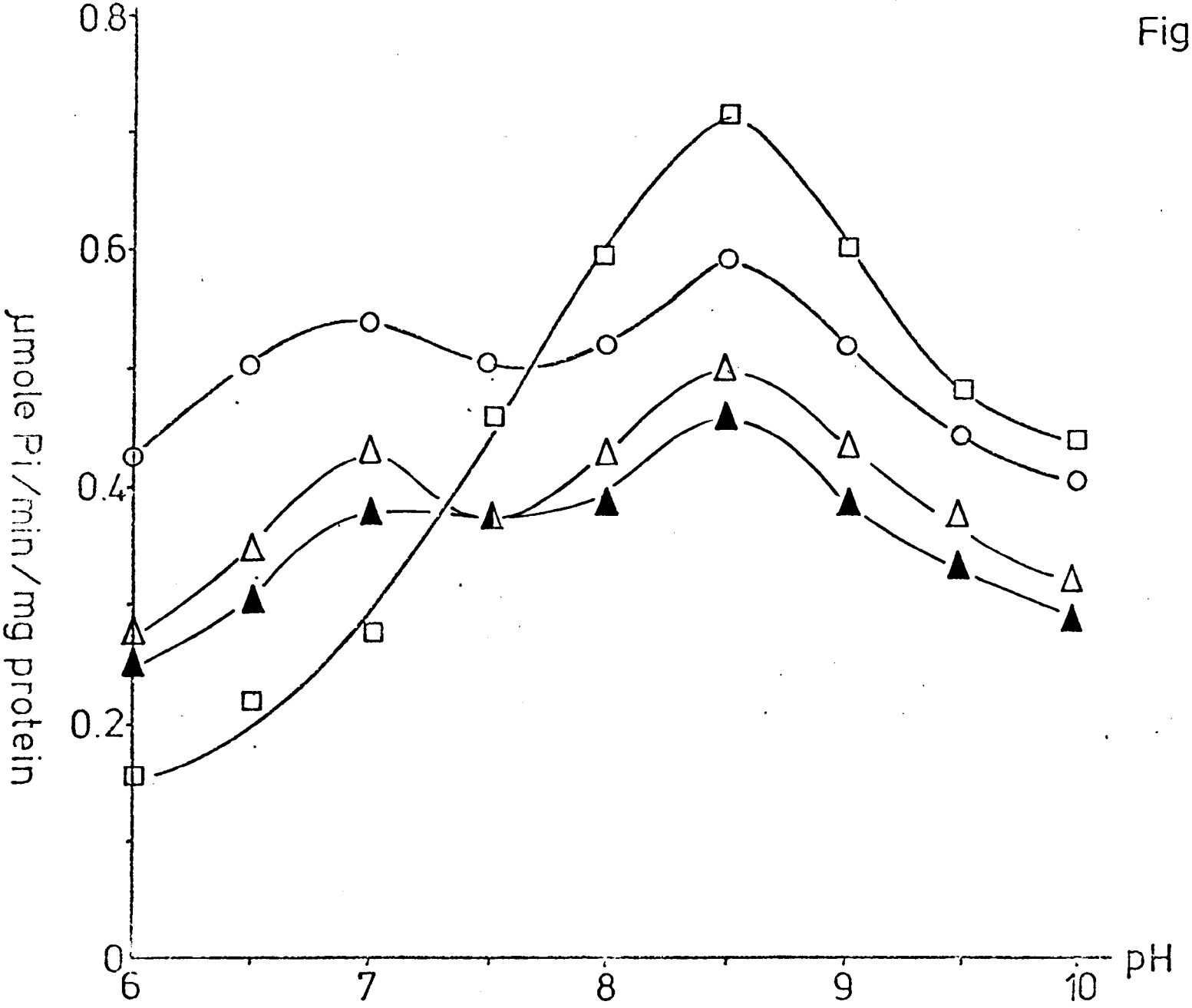
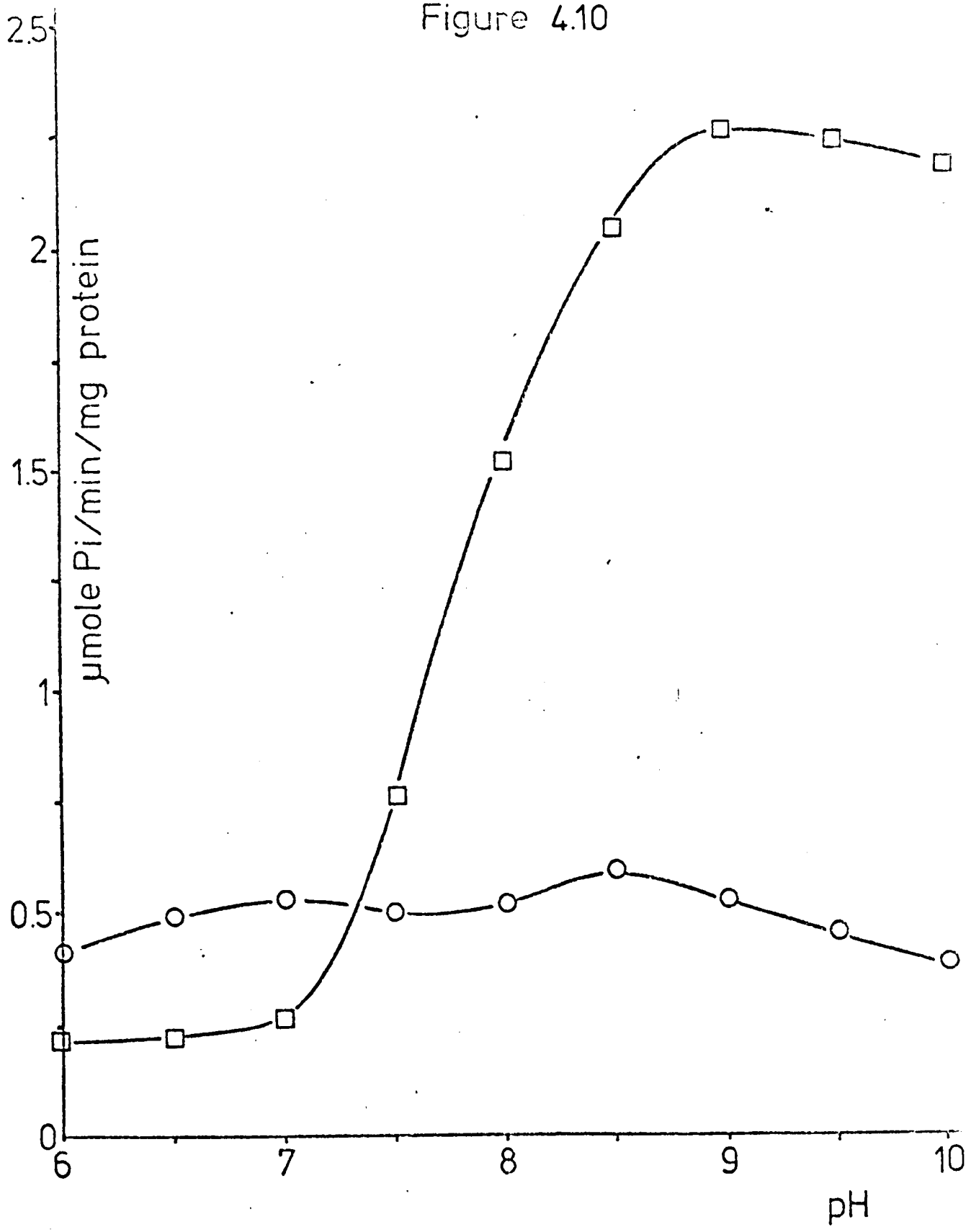


Figure 4.10



The effect of pH on the mitochondrial ATPase in S. lipolytica grown in the fermenter on different substrates. Mitochondria were prepared using the Braun shaker and gradient purified before use.

O - O Stationary phase on 0.5% (v/v) ethanol.

□ - □ Logarithmic phase on 10.0% (w/v) glucose.

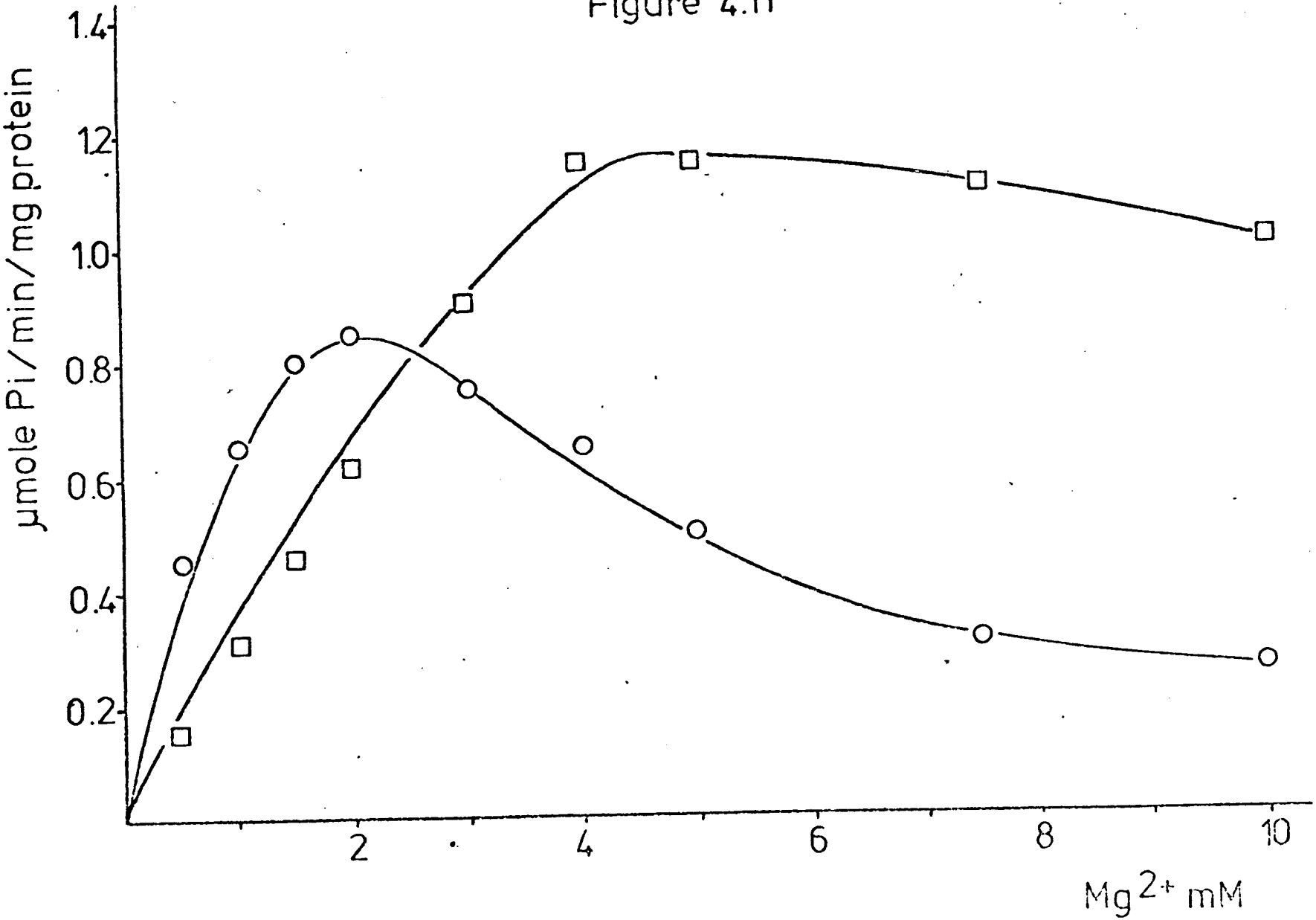
FIGURE 4.11

The effect of Mg^{2+} on the mitochondrial ATPase in S. lipolytica. Cells were grown in the fermenter to stationary phase on 0.5% (v/v) ethanol. Mitochondria were prepared using the Braun shaker and gradient purified before use.

O - O pH 9.0.

□ - □ pH 7.0.

Figure 4.11



of the S. lipolytica enzyme was never greater than 0.8 - 1.0 μmole Pi/min/mg protein, even in the pH range 8.0 - 10.0. Addition of DNP (0.1 mM) to mitochondria isolated by the snail enzyme procedure had very little effect on the ATPase activity over the entire range from pH 6.0-10.0.

Growth of S. lipolytica on n-tetradecane (0.2% v/v) affects the pH profile of the mitochondrial ATPase in early stationary phase cells (Figure 4.9). There is a maximum at pH 8.5 only, but again the specific activity is relatively low at around 0.7 μmole/Pi/min/mg protein. The characteristics of this ATPase in cells grown to early stationary phase on n-pentadecane are similar to those of the n-tetradecane grown cells.

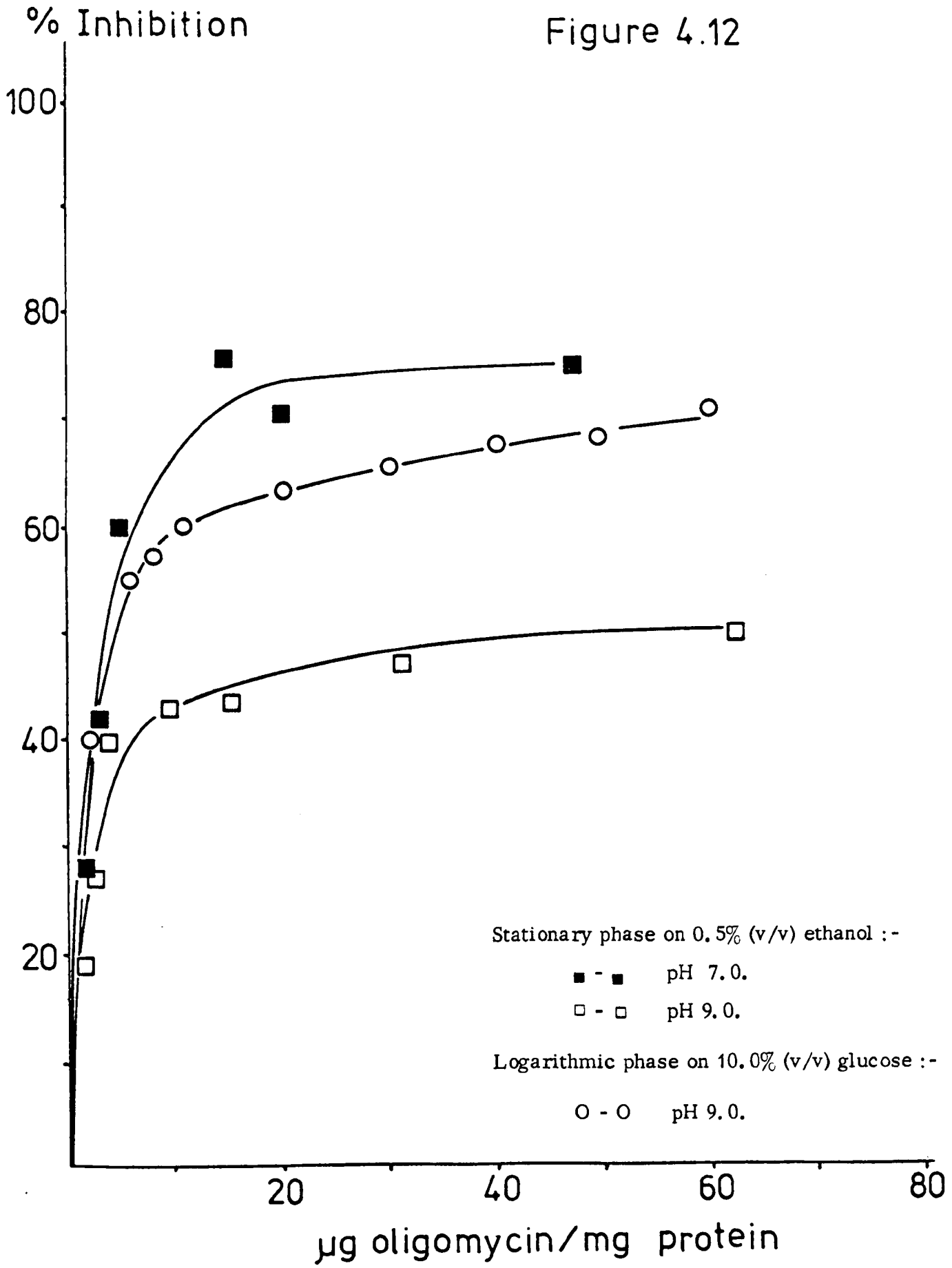
S. lipolytica harvested during the logarithmic phase of growth on 10% (w/v) glucose had one well defined maximum in the pH profile of the mitochondrial ATPase activity. This was at pH 9.0 (Figures 4.10) and, in contrast to the enzymes in S. lipolytica cells grown on ethanol or on n-alkanes, the specific activity was in the range 2-2.5 μmole Pi/min/mg protein.

The S. lipolytica ATPase was entirely dependent on the presence of Mg^{2+} ions for activity (Figure 4.11). For ethanol grown cells the optimal Mg^{2+}:ATP ratios were 1:1 at pH 7.0 and 1:2 at pH 9.0. In contrast to the S. cerevisiae enzyme (Figure 3.22) the specific activities of the S. lipolytica ATPase were both in the same range (0.8-1.2 μmole Pi/min/mg protein) even at optimal Mg^{2+}: ATP ratios. In both n-alkane and glucose grown cells of S. lipolytica the optimal Mg^{2+}: ATP ratio at pH 9.0 was also 1:2.

Inhibition by oligomycin is a characteristic feature of the mammalian (Lardy et al, 1958) and of the yeast (Kovac et al, 1968) mitochondrial ATPases Even promitochondria isolated from Saccharomyces sp. grown under conditions of anaerobiosis and glucose repression retain a mitochondrial ATPase activity and this is still relatively sensitive to inhibition by oligomycin (Criddle and Schatz, 1969; Watson et al, 1970, 1971). In mitochondria isolated from S. cerevisiae grown aerobically on ethanol, the ATPase activity at either pH 7.0 or pH 9.5 can be inhibited to approximately 90% by high concentrations of oligomycin (Figure 3.23).

The sensitivity of the mitochondrial ATPase activity in cells of S. lipolytica to inhibition by oligomycin (Figure 4.12) presents a contrasting situation. First, the activity at pH 7.0 was more easily inhibited by oligomycin than the activity at pH 9.0. Second, maximal inhibition by oligomycin was much less than in

Figure 4.12



The effect of oligomycin on the mitochondrial ATPase in S. lipolytica cells grown on ethanol or on glucose in the fermenter. Mitochondria were prepared using the Braun shaker and gradient purified before use.

TABLE 4.3.

Effect of oligomycin on the mitochondrial ATPase from S. lipolytica grown on different substrates.

	Substrate	pH of Assay	µg oligomycin/mg protein			µg oligomycin/mg protein at 50% Inhibition.
			2	10	50	
			Percentage Inhibition			
1.	Ethanol	7.0	30	67	74	3.5
2.		9.0	28	43	49	50
3.	n-Tetradecane	7.0	45	81	82	3
4.		9.0	38	58	62	5
5.	Glucose	9.0	39	59	68	5

Notes.

1. These results are derived from Figure 4.12.

S. cerevisiae in either case, as shown by the plateaux on the inhibition curves (Table 4.3).

The ATPase of mitochondria isolated from S. lipolytica grown on n-tetradecane gave the same pattern of oligomycin inhibition as that of the enzyme isolated from ethanol grown cells. However, the activities at pH 7.0 and at pH 9.0 were somewhat more sensitive to oligomycin (Table 4.3).

The response of the ATPase from n-tetradecane grown cells to oligomycin at pH 9.0 is virtually identical to that of the enzyme from S. lipolytica harvested in log phase during growth on 10% (w/v) glucose (Figure 4.12). No assays were done at pH 7.0 in this case. The ATPase was more sensitive to oligomycin than the corresponding activity in ethanol grown cells with a maximal inhibition of 60-70% (Table 4.3).

DISCUSSION.

Several species of microorganisms are able to utilise hydrocarbons as sole carbon scource (Klug and Markovetz, 1971; Einsele and Fiechter, 1971). The increasing interest over the last few years in hydrocarbon oxidation reactions is primarily related to the rational use of petroleum and gaseous hydrocarbons. Production of biomass from growth of microorganisms on aliphatic petroleum fractions has given rise to expanded prospects for industrial protein production and the biosynthesis of other compounds.

The question arises whether the ability of microorganisms to grow on hydrocarbons is inducible or constitutive. Generally the oxidation of hydrocarbons has been found to be inducible in the presence of hydrocarbon (van der Linden and Thijsse, 1965). Induction of hydrocarbon oxidation by non-hydrocarbon substrates has also been reported (van Eyk and Bartels, 1968) but glucose was found to be a strong repressor of hydrocarbon oxidation. Species of Candida yeast have been used in a number of growth studies (Klug and Markovetz, 1967a; Munk et al, 1969a) and these will utilise n-alkanes of chain length C_{10} - C_{20}. Azoulay et al (1964) were able to select relatively high yielding strains of Candida lipolytica by repeated culture on n-alkanes. Duvnjak et al, (1970) indicated that the ability to degrade n-tetradecane may be constitutive in some strains of Candida tropicalis.

By extrapolation of the data of McAuliffe, (1963) for short chain n-alkanes, Johnson (1964) showed that only extremely small amounts of the n-alkanes of chain length C_{10} or more can be maintained in aqueous solution. However, since there is a change from true solubility to accommodation beginning with n-alkanes of chain length C_{11} (McAuliffe, 1969), C_{12} - C_{18} n-paraffins are all present in water at about the same concentration. This low solubility of n-alkanes leads to the consideration of several mechanisms for the uptake of liquid paraffins by yeast. Growth may be supported by dissolved (or accommodated) substrate or assimilation may only occur when the cells are in direct contact with droplets of n-alkane in suspension. An intermediate situation may also operate in which both processes are used. In any case, growth will be influenced by the solubility and degree of emulsification of the hydrocarbon. The extent of dispersion of the n-alkane governs the amount of surface area available for direct contact and controls the rate of transfer of the substrate into aqueous solution (Ladd, 1956).

A model assuming linear characteristics for yeast grown on n-alkanes has been constructed by Erickson et al, (1969), and McLee and Davies, (1972) attribute linear growth of Torulopsis sp. on n-paraffins to be due to a finite surface area being available for direct contact between cells and hydrocarbon. Aiba et al, (1969 a, b) consider that the uptake of n-alkanes by the yeast Candida guilliermondii is again by direct contact and have proposed a model for growth based on estimates of the interfacial area between the cells and the hydrocarbon droplets.

Yeasts are able to grow logarithmically on n-alkanes under the correct culture conditions (Wagner et al, 1969; Moo-Young et al, 1971). A mechanistic model can be presented (Moo-Young and Shimizu, 1971) which again assumes that growth of the yeast is governed by the extent of attachment of the cells to suspended paraffin droplets. Growth rates will increase with increasing dispersion of the n-alkane by increased agitation. Yeast cells growing on n-alkanes are found clinging to droplets of emulsified substrate (Bos and de Boer, 1966). When the droplets were the same size as the yeast cells there was a decrease in growth rate (Bakhius and Bos, 1969).

Johnson, (1964) proposed that the uptake of n-alkanes was non-specific and did not rely on permeases in order to traverse the cell membrane. This implies that hydrocarbons would be transported even though they could not be metabolised. Ludvik et al, (1968) have determined that in C. lipolytica n-paraffins penetrate the cell wall and accumulate at the cell membrane. Yeast grown on hydrocarbon show morphological changes compared with those grown on glucose. Alkane molecules penetrate the cell wall as a whole through passive diffusion, and then are actively transported through the plasma membrane (Einsele and Fiechter, 1971). However, protoplasts of C. lipolytica lose the ability to oxidise n-hexadecane (Volfova et al, 1967). This result may be contrasted with evidence that protoplasts of C. tropicalis retain the ability to oxidise n-alkanes (Lebeault et al, 1969 a, b). Munk et al, (1969b) have established that there is some specificity in the selection of n-paraffins transported into the cell since those not supporting growth were not taken up.

An argument in favour of direct uptake of n-alkanes by yeast cells is that if uptake was only through the dissolved or accomodated state then this would limit the exponential phase of growth. However, if the surface area of the hydrocarbon droplets is high enough then a high transfer rate to the cells may be maintained even if the solubility of the n-alkane is low. Goma et al,

(1973) have shown by kinetic analyses of growth of C. lipolytica on mixtures of n-alkanes that the more soluble members are utilised first. They conclude that there is little probability of a direct uptake mechanism occurring. In the present studies growth on n-decane was initiated more rapidly than on n-tetradecane or on n-hexadecane, (Figure 4.4). These data may indicatethat in S. lipolytica, strain YB-423, n-alkane uptake is primarily through the dissolved (or accommodated) state. However, if this were exclusively the case the maximal growth rates seen during log phase could be expected to differ, but this is not so. There may in this phase of growth be some uptake by direct contact. However, these results are in line with the solubility data of McAuliffe, (1969) and in broad agreement with Goma et al, (1973). In this strain of S. lipolytica it is not certain whether n-alkane oxidation is inducible or constitutive. Should it be inducible it is probable that the more soluble paraffin would be the more effective inducer and would tend to promote faster growth. In order to resolve this situation studies of the growth parameters of S. lipolytica on mixtures of n-alkanes should be done.

When the concentration of hydrocarbon substrate (n-tetradecane) was increased the lag phase of growth was also prolonged (Figure 4.3). This is evidence that other factors such as the degree of emulsification of the n-alkane, and extent of agitation and/or aeration of the medium must be taken into account (Einsele and Fiechter, 1971). It is possible that in S. lipolytica uptake of n-alkane into the cells may proceed through all possible mechanisms. Assimilation may be through the dissolved state in the initial phases of growth and then by direct contact in log phase. An increase in the initial substrate concentration may require a corresponding increase in agitation and/or aeration in order to maintain similar kinetics in the early stages of growth in batch culture.

Yeast grown on n-alkanes in liquid culture have a high oxygen demand per unit cell mass (Darlington, 1964; Johnson, 1964). Critical oxygen concentrations in culture media may exert control over various cell components especially if n-paraffins are used as substrates (Klug and Markovetz, 1969; Karban et al, 1969), Einsele et al, (1972) have followed the oxygen uptake of C. tropicalis during growth on glucose and on n-hexadecane and they conclude that the specific rate of respiration is the same in both cases (200 nmoles per min per mg dry weight). This compares well with the data shown in Figure 4.2 for S. lipolytica on ethanol. If excess oxygen is supplied, rates of synthesis of cellular material may be limited by the respiratory activity. According to Payne, (1970) hydro-

carbon substrates have 3-4 times more electrons available to combine with oxygen than glucose or ethanol. Since growth rates on hydrocarbons are slower by a factor of at least two-fold it must be concluded that the respiratory efficiency of the cells is a limiting step for growth. This indicates that a good deal of the energy of the hydrocarbon substrate, which is available for conversion into biomass, is wasted as heat.

The growth rates shown in these Results for S. lipolytica on glucose, on ethanol and on n-alkanes are in the same range as those found by other workers. It is difficult to compare results directly since the growth rate depends on several factors previously discussed (Karban et al, 1969; Einsele and Fiechter, 1971; Moo-Young et al, 1971). Growth yield experiments were attempted in batch culture according to the method of Kormancikova et al, (1969). Measureable growth of S. lipolytica occured on casein hydrolysate alone, which was also preferentially utilised before the n-alkanes, hence these experiments were discontinued. However, the yields of cells with n-alkanes as sole substrate are extremely low especially when compared to molar yields on glucose or ethanol. The proposals of Stouthamer and Bettenhausen, (1973) may be applicable in this context.

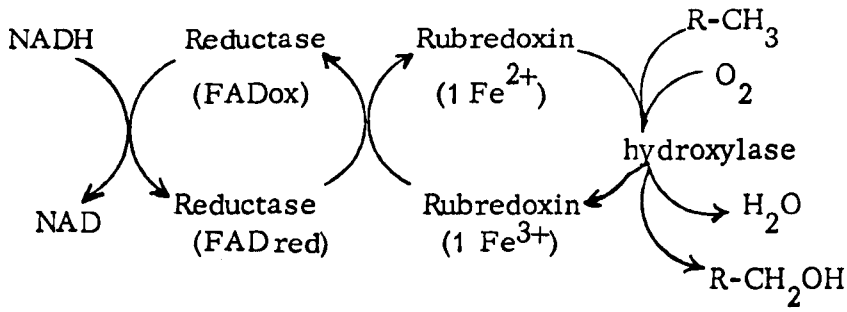
Microorganisms capable of growing on hydrocarbons as sole carbon scource include several species of yeasts and bacteria (Karban et al, 1969; Klug and Markovetz, 1971), and it is necessary to give some consideration to the mechanism of oxidation of aliphatic hydrocarbons. The breakdown of aromatic and heterocyclic molecules is considered by Karban et al, (1969) and Jerina, (1973). The major pathway of biological oxidation of n-alkanes is by initial attack at a terminal carbon atom, resulting in the formation of the corresponding primary alcohol. This leads to the successive production of the aldehyde and the fatty acid which is then presumably metabolised by β-oxidation. Other mechanisms do however exist (Allen et al, 1971) but these also involve initial attack by oxygen with a fatty acid as final product. Stewart et al, (1960) observed $^{18}O_2$ incorporation when Micrococcus sp. was grown on hexadecane and concluded that the primary attack on the alkane involved both molecular oxygen and an oxygenase. The problem of activation of saturated hydrocarbons has been the subject of much discussion (Shilov and Shteinman, 1973). The non-catalysed reaction of n-paraffins with oxygen in solution usually proceeds through a degenerate, branched chain, free radical mechanism. This is subject to variations as transition metals are used as catalysts in order to activate either oxygen or hydrocarbon.

Biological oxidation of hydrocarbons, including saturated species, is brought about with the participation of mixed function oxidases or mono-oxygenases. the reaction proceeds simultaneously with the oxidation of NAD(P)H according to the scheme:-

$$NAD(P)H + H^+ + R - CH_3 + {}^{18}O_2 \longrightarrow NAD(P) + R - CH_2{}^{18}OH + H_2{}^{18}O$$

A characteristic feature of enzymic oxidation is the preservation of configuration during oxidation at the C-H bond (Hamberg and Bjorkhem, 1971). It may therefore be assumed that the reaction proceeds without the formation of free radicals.

Baptist et al, (1963) demonstrated that cell free extracts from Pseudomonas oleovorans grown on n-octane form the corresponding primary alcohol in a reaction requiring both NADH and molecular oxygen. This alkane hydroxylase system also involves rubredoxin and a rubredoxin reductase which are present in electron transfer from NADH to the hydroxylase (Peterson et al, 1967; Coon et al, 1972).



More recently, Cardini and Jurtshuk, (1970) have obtained from Corynebacterium sp. another type of alkane hydroxylase which involves cytochrome P450 and a flavoprotein. In this case alkanes may be converted into primary alcohols via initial formation of a hydroperoxide. The characterisation of a multi-component hydroxylation system in Pseudomonas putida grown on camphor has been described by Gunsalus and co-workers (Gunsalus et al, 1972a,b). This contains a specific cytochrome $P450_{cam}$ as terminal hydroxylase; putidaredoxin and a flavoprotein arranged to transfer electrons from NADH as in the P. oleovorans system. (Gunsalus et al, 1973).

Candida tropicalis grown on n-alkanes contains cytochrome P450 (Gallo et al, 1971; Lebeault et al, 1971), as the terminal component of the paraffin hydroxylating system. This is a mixed function oxidase, requiring NADPH and molecular oxygen for activity (Duppel et al, 1973). This yeast cytochrome P450 has many properties similar to the mammalian drug hydroxylation system (Coon et al, 1972) and is also located in the microsomal fraction of the cells (Gallo et al, 1971). The n-alkane hydroxylation enzymes are now thought to be

inducible in Candida yeast (Gallo et al, 1973b; Liu and Johnson, 1971). In active microsomal particles this hydroxylase system is associated with alcohol and aldehyde dehydrogenases and these enzymes are also present in the mitochondria (Lebeault et al, 1970 a, b; Gallo et al, 1973 a, b). Mitochondrial fragments of C. tropicalis contain an ATP-dependent trans-hydrogenase (Gallo et al, 1973 b). This would explain previous reports that ATP increases NAD reduction by impure n-decane which resulted in the postulation of an n-alkane dehydrogenase in this yeast (Lebeault et al, 1970c). Growth on n-alkanes also causes a considerable increase in the cellular levels of NADPH-cyctochrome c reductase, catalase and isocitrate lyase relative to growth on glucose. The levels of other enzymes present in the tri-carboxylic acid and glyoxalate cycles are, however, unchanged (Gallo et al, 1973 a). These authors also indicate that growth on n-alkanes increases the level of b-type cytochromes in the cell and induces the formation of one or more soluble heme like substances.

Cells of S. lipolytica grown on ethanol achieve higher levels of cytochrome aa_3 than those of S. cerevisiae, strain D22 (Table 4.1). S. lipolytica grown on any substrate, including n-alkanes, also has a relatively broad cytochrome b absorption in the α-region of the reduced minus oxidised spectrum (Figures 4.5 - 4.8). The cytochrome profile of S. lipolytica mitochondria compares with that found for Candida utilis grown on ethanol (Grimmelikhuijzen and Slater, 1973). Growth of S. lipolytica on n-alkanes produces an increase in absorption due to b-type cytochromes in the cell in line with the observations of Gallo et al, (1973a). There was also a depression in the content of cytochromes c and aa_3 (Table 4.1) but no evidence for any other heme like substance with absorption peaks between 600 nm and 700 nm. Interpretation of the variations in cytochrome contents of S. lipolytica grown on different substrates is difficult since different growth conditions will produce changes in the cytochrome profile of the cells (Figure 4.5). For S. lipolytica cells grown in batch culture on n-tetradecane there was some repression of cytochrome contents in log phase cells relative to stationary phase cells. Although the cytochromes in S. lipolytica are not as much affected by substrate repression, e.g. by glucose, as those in S. cerevisiae (Figure 4.6). The possibility exists that the variation in cytochrome content of n-paraffin grown cells during growth may be due to external conditions rather than to metabolic changes within the cell. There may be variations in the oxygen tension and/or availability of the substrate depending on the hydrocarbon concentration. However the large amount of fatty acids, produced from n-alkane

oxidation, which will be present in the cell during log phase may directly or indirectly affect the mitochondrial membranes. This may result in repression of the respiratory enzymes. This point will be further discussed in the next Chapter. Growth of cells on odd-numbered or even-numbered hydrocarbons produces essentially no differences between the cytochrome profiles of stationary phase cells (Figures 4.7 and 4.8).

Cytochrome P450 is characterised by the intense absorption at 450 nm of the reduced carbon monoxide compound (Omura and Sato, 1964), and its further spectral characteristics have been described (Nishibayashi et al, 1968; Lemberg and Barrett, 1973). From these data on S. lipolytica (Figures 4.7 and 4.8) there is no spectroscopic evidence for the existence of cytochrome P450. The Soret bands are at 415 nm in the oxidised, and 420 nm in the reduced form of this cytochrome and these are masked by the mitochondrial species. Carbon monoxide difference spectra were attempted on cells grown to stationary phase on n-tetradecane but only the typical cytochrome a_3 difference spectrum was seen (Chance, 1957) with a peak at 430 nm and a trough at about 445 nm. No peak was seen at 450 nm. It is possible however that the larger absorption due to b-type cytochromes, present in the reduced minus oxidised spectrum of S. lipolytica grown on n-alkanes relative to that in ethanol grown cells may in part be due to induced synthesis of cytochrome P450, which has a reduced α-band at about 555 nm.

In common with the liver microsomal hydroxylation system (Coon et al, 1972) the C. tropicalis enzyme complex which promotes the terminal oxidation of long chain n-alkyl groups has been isolated in an apparently soluble form (Duppel et al, 1973). The system has been resolved into three components; cytochrome P450, NADPH-cyctochrome P450 reductase, and a heat stable lipid fraction, all of which are necessary, along with NADPH and O_2, to obtain hydroxylase activity. Although cytochrome c is used for convenience of assay of NADPH cytochrome c reductase (present in the microsomal fraction from C. tropicalis) it is presumed that cytochrome P450 is the natural acceptor (Duppel et al, 1973; Gallo et al, 1973 a, b). Mechanisms have been postulated for the action of the liver cytochrome P450 (Coon et al, 1972) and P. putida cytochrome $P450_{cam}$ (Gunsalus et al, 1972[a,b]/1973). Both involve the transfer of two electrons from NAD(P)H to cytochrome P450 in order to activate bound molecular oxygen to a superoxide species and enable it to attack the substrate.

n-Alkane hydroxylase activity in purified microsomal preparations of C. tropicalis isspecific for NADPH and this system is closely associated with NAD-dependent alcohol and aldehyde dehydrogenases. This would indicate that conversion of the n-alkane to fatty acid takes place in the same area. In Candida intermedia however, Liu and Johnson (1971) have shown that the enzymes catalysing the oxidation of n-decane to decanoic acid are located in the cell membrane. The actual site of these enzymes may well vary from one species of yeast to another. Complementary evidence indicating that in Candida sp. n-alkane metabolism proceeds through a fatty acid or fatty acid intermediate is obtained from mutants unable to grow on n-tetradecane (Bassel and Mortimer, 1973).

In view of the differences between cytochrome profiles of S. lipolytica grown on n-alkanes and cells grown on ethanol, attempts were made to prepare intact mitochondria, capable of respiratory control, in order to study the process of oxidative phosphorylation. Volfova et al, (1967) have prepared protoplasts from C. lipolytica grown on glucose and Lebeault et al, (1969) have obtained protoplasts from C. tropicalis grown on n-tetradecane, again using helicase to digest the yeast cell wall. Attempts were made to prepare protoplasts from S. lipolytica as described in Chapter 3 for S. cerevisiae or using the conditions of Lebeault et al, (1969). Protoplasts were only produced inthe case of ethanol grown cells harvested in the logarithmic phase of growth. The mitochondria obtained did not show respiratory control. In order to provide more information on the properties of mitochondria present in S. lipolytica cells grown on various substrates, measurements were made of respiratory enzymes and of the mitochondrial, Mg^{2+}-dependent ATPase activity in each case. The activities of the respiratory chain components have been related to the cytochrome profiles and also to the fatty acid content of the mitochondrial membranes by means of Arrhenius plots. These are discussed in the following Chapter.

There is a noticeable contrast between the characteristics of the mitochondrial, Mg^{2+}-dependent ATPase in S. lipolytica (Figures 4.9 - 4.12) and that in S. cerevisiae, strain D22 (cf. Chapter 3). In S. lipolytica grown on ethanol the specific activity of the ATPase is very low, less than 1 μmole Pi/min/mg protein throughout the pH range 6 - 10, with maxima at pH 7.0 and 8.5. Uncouplers have little effect on the specific activity of this enzyme, even in mitochondria prepared using the snail enzyme method. Cells grown on n-tetradecane through to those grown on glucose show an increase in specific activity at pH 8.5 - 9.0.

It is possible that S. lipolytica contains some kind of ATPase inhibitor similar to bovine heart mitochondria (Pullman and Monroy, 1963). Alternatively there may be less ATPase incorporated into the mitochondria. Growth on glucose may cause increased synthesis of ATPase or a decrease in the activity of the ATPase inhibitor (Vignais et al, 1973). Estimation of the mitochondrial ATPase by aurovertin titration (Lardy and Lin; 1969; Van de Stadt et al, 1974) may clarify this situation.

In common with S. cerevisiae, the S. lipolytica mitochondrial ATPase is Mg^{2+}-dependent (Figure 4.11). Even at optimal Mg^{2+}:ATP ratios the specific activities at pH 7.0 and pH 9.5 are both of the same order, around 1 µmole Pi/min/mg protein. These low specific activities are surprising in view of the relatively high cytochrome contents and the correspondingly higher activities of the respiratory enzymes.

In contrast to other mitochondrial ATPases the S. lipolytica enzyme is not completely inhibited by oligomycin (Figure 4.12). The titration curves reach plateaux at between 40% and 70% inhibition, even at high concentrations of the antibiotic. The ATPase from glucose grown cells is somewhat more sensitive to oligomycin. The controlled effect of oligomycin may be due to a change in the number or type of binding sites for it, relative to those in other systems eg. S. cerevisiae. Alternatively there may be variations in the co-operative interactions brought about by oligomycin once bound, which affect the ATPase. Separation of the ATPase components after the fashion of Tzagoloff, (1969 a.b) coupled with selective reconstitution experiments (Schatz, 1968) may provide the reason for this incomplete inhibition by oligomycin.

CHAPTER 5 Fatty Acids and Sterols of Saccharomyces cerevisiae and Saccharomycopsis lipolytica.

INTRODUCTION

Drug or antibiotic resistant strains of S. cerevisiae (Griffiths, 1972) may be assumed to have mutations resulting in variations in the makeup of the inner mitochondrial membrane. The mutation may be specific to mt-DNA and/or the resulting structural change restricted to the binding site of the drug or antibiotic e.g. Class 2 OL^R mutants (Avner and Griffiths, 1973a). In contrast, Class 1 OL^R strains have been classified as "general membrane mutants" and probably result from simultaneous nuclear and mitochondrial genetic contributions (Avner and Griffiths, 1973b).

From the previous Chapters it may be concluded that Class 2 TET^R and uncoupler resistant mutants have more widespread changes in the properties of the mitochondrial inner membrane. This consists of a complex of phospholipids and proteins (Review, Chapman and Leslie, 1970) and the site of interaction with any effective agent would have contributions from these constituents. Certainly oligomycin will bind to phospholipids (Palatini and Bruni, 1970) and the site of action of DCCD involves proteolipid (Cattell et al, 1971).

The mitochondrial membranes may have phospholipid bilayer structure (Hsia et al, 1972) and lipids are necessary for mitochondrial energy coupling (Tinberg et al, 1972). These authors suggest that they undergo physical changes at the molecular level coincident with variations in the energy state of the mitochondrion. The possibility exists therefore that whilst specific mutation to drug resistance by S. cerevisiae is more likely to result in an altered membrane protein, changes in lipid content may also occur. Accordingly the fatty acid profiles of selected drug resistant mutants have been examined.

Discontinuities in the Arrhenius plots of membrane bound enzymes have been interpreted as being due to phase changes in the lipid components of the membrane which induce conformational changes in the enzyme proteins (Raison et al, 1971 a, b). Arrhenius plots of respiratory enzymes in S. cerevisiae mitochondria show discontinuities which are correlated with the fatty acid profile.

The fatty acids of S. cerevisiae have been contrasted with those of S. lipolytica. Significant alteration in the lipid composition of S. lipolytica occurs between cells grown on ethanol and n-alkanes. Arrhenius plots of respiratory enzymes in mitochondria isolated from these cells are compared and related to the corresponding fatty acid profile.

Resistance to certain antibiotics is associated with changes in the sterol content of S. cerevisae cells (Woods, 1971; Molzahn and Woods, 1972); accordingly the sterols present in selected drug resistant mutants have been examined. The presence of sterols in the inner mitochondrial membrane affects the transition temperature of the lipid constitutents (Chapman and Leslie, 1970). The sterols of S. cerevisiae and S. lipolytica have therefore been compared in the light of the corresponding Arrhenius plots.

METHODS AND MATERIALS

Growth of S. cerevisiae.

The cells were grown in 500 ml medium in 2 ltr. conical flasks which were baffled for efficient oxygenation. The medium contained 0.5% (w/v) yeast extract; mineral salts (Wickerham, 1946) and 0.1 g adenine sulphate per litre of distilled water. The pH was adjusted to 5.5 and the carbon scource was either ethanol (0.5% v/v) or glucose (5.0% w/v). A 1.0% (v/v) sample of a starter culture previously grown on ethanol (0.5% v/v) was used as inoculum. Growth conditions were as previously described for the measurement of growth and respiratory activity of S. cerevisiae on ethanol (Chapter 2).

Yeast were also grown in 10 ltr batch culture in New Brunswick fermenters. The carbon scource was always ethanol (0.5% v/v) and the composition of the medium was as above. Tri-butyl citrate was used as anti-foaming agent at a concentration of 0.1 - 0.2 ml per ltr. The growth conditions were as described in Chapter 3.

Growth of S. lipolytica.

S. lipolytica (strain YB-423) was grown on various substrates in 10 ltr batch cultures in the New Brunswick fermenter. The culture medium, together with the growth conditions have been previously described in Chapter 4. In this case the carbon scources were ethanol (0.5% v/v), glucose (5.0% w/v) or n-alkane (0.2% v/v).

Preparation of Mitochondria (Braun shaker).

A description of this procedure is given in Chapter 2. In every case cells harvested in early stationary phase were used. The mitochondria obtained were always gradient purified before use.

Estimation of Fatty Acids.

1.0 - 2.0 g wet weight of yeast cells (harvested in early stationary phase) or 20 - 40 mg mitochondria were resuspended in 3.0 - 5.0 ml of 40% (w/v) potassium hydroxide and refluxed for 4 hr in order to saponify the lipids present. The mixture was extracted at least three times with 20 - 30 ml petroleum ether (b.p. 40 -60^{o}C).

These ether extracts contained the sterol constitutents of the yeast material and were either discarded or kept as necessary.

The aqueous phase was then acidified with concentrated HCl, again extracted with petroleum ether and the pooled extracts dried over anhydrous sodium sulphate, for 3 - 12 hr. The ether was removed under vacuum and 3.0 - 5.0 ml of methanol/HCl (95/5% v/v) added.

Methyl esters were formed by refluxing for 2 hr at 60° C. They were extracted into 5.0 ml n-hexane and determined by gas-liquid chromatography using a Perkin-Elmer F-11 instrument. An analytical column (length 2 m, diameter 1/8 in) containing 20% diethylene glycol succinate on Chromosorb W HMDS (mesh 80-100) was used at 175°C with nitrogen as carrier gas. Relative percentages of fatty acids were determined by measurement of peak areas. Calibration was by methyl ester standards.

Estimation of Sterols.

The pooled, initial petroleum ether extracts from fatty acid estimation were evaporated under vacuum and the sterols redissolved in 5 ml n-heptane. The absorption spectrum was recorded using a Unicam SP 1800 scanning spectrophotometer with 1 cm light path cells.

Alternatively, sterols were assayed according to the method of Breivik and Owades, (1957), as described by Woods, (1971), except that non-alcoholic 40% (w/v) potassium hydroxide was used. Extraction of the saponified mixtures was directly into 10 ml n-heptane and this extract was examined spectrophotometrically as above.

Succinate Oxidase and NADH Oxidase Assays.

Oxygen uptake was measured using a Rank electrode (Rank Bros., Bottisham, Cambridge, U.K.) equipped with a Churchill refridgerated thermostat (Churchill Instrument Co. Ltd., Perivale, Middlesex, U.K.). 2 ml of air-equilibrated buffer containing 50 mM potassium phosphate, pH 7.4 was used for each assay. 50 - 200 µl of mitochondrial suspension (0.5 - 1.5 mg protein) was added and, after a further 1 min, 0.4 mM ADP, followed by either NADH (2.5 mM) or succinate (20 mM) as substrate.

Succinate Dehydrogenase Assay.

A Cary 14 spectrophotometer fitted with a Churchill refridgerated thermostat was used to assay this enzyme. Accurate temperature measurement was by means of a thermocouple (± 0.1°C) which fitted directly into the cuvette (1 cm light path). The reaction mixture contained, in a final volume of 1.1 ml, 50 mM potassium phosphate buffer, pH 7.4, 50-100 μg mitochondrial protein, 25 mM succinate and 2 mM KCN. Incubation was for 5 min and the reaction was started by the rapid addition of 0.05 mM DCPIP and 0.5 mM PMS. The decrease in absorbance of DCPIP at 600 nm was followed (e_{mM} = 21; King and Howard, 1967). A current of dry nitrogen was passed through the cell chamber to prevent condensation at the lower temperatures.

Dry weight and Protein Estimations.

Dry weights of intact cells were measured by filtration onto Whatman GF/C glass fibre paper (2.4 cm diameter) and dried in an oven at 110-120°C for 2-3 days. Protein estimations were by the Lowry procedure. (Lowry et al, 1951).

Materials

ADP, PMS, DCPIP and NADH were obtained from Sigma (Sigma (London) Chemical Co., Kingston-on-Thames, U.K.). Methyl ester standards were obtained either from Sigma or from B.D.H. (B.D.H. Chemicals Ltd., Poole, Dorset, U.K.). All other chemicals were of Analytical Reagent grade where available.

RESULTS

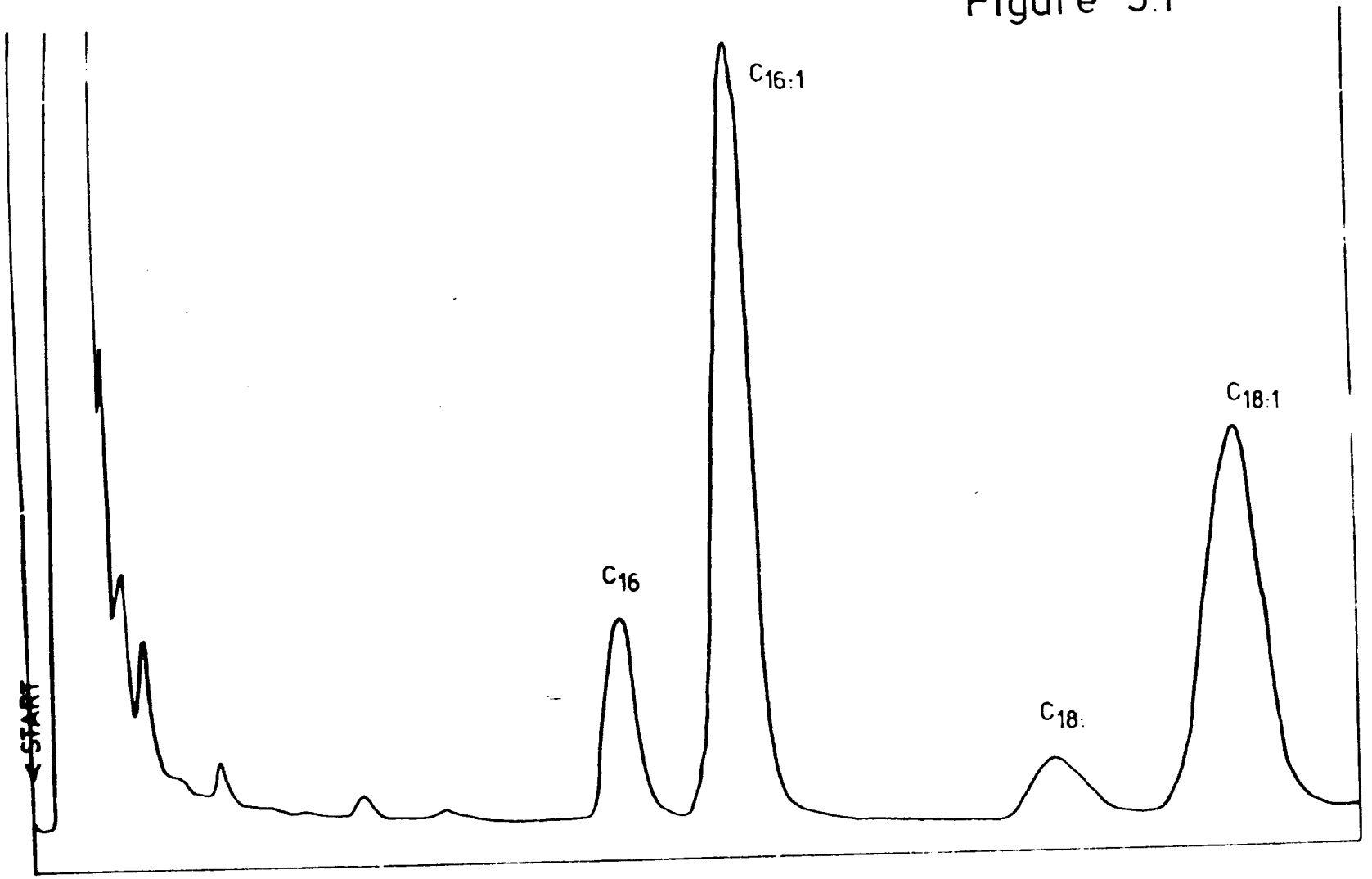
The fatty acid compositions of aerobically grown, intact cells of S. cerevisiae, strain D22 and selected drug or uncoupler resistant mutants have been examined. All strains contain a high percentage of unsaturated fatty acids (> 80%). The cells were extremely rich in palmitoleic acid ($C_{16:1}$) which made up 45% of the total cellular fatty acids with oleic acid ($C_{18:1}$) accounting for about 35% (Figure 5. 1). Only small amounts of the saturated acids, palmitic (C_{16}) and stearic (C_{18}) were found. This distribution of fatty acids has been well documented for S. cerevisiae cells (Watson et al, 1971). The differences in relative fatty acid content between the wild type and any of the mutants are not significant (Table 5. 1). There may be quantitative differences between one strain and another but these were not tested for. The mutants listed in Table 5. 1 are all Class 2, specific types. Similar results (not shown) were also obtained for the Class 1, OL^R mutant, D22-B9.

The fatty acid profile of S. cerevisiae, strain D22, is compared with that of S. lipolytica cells grown on different substrates. Under all conditions of aerobic growth, with glucose, ethanol or n-alkane as carbon scource, these cells contain 80 - 90% of their total fatty acids as unsaturated species (Table 5. 2). On most substrates the major components were oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acids. In S. lipolytica grown on glucose or on ethanol the ratio of these acids was approximately 1:1. These made up about 80% of the cellular fatty acids with small amounts of palmitic and palmitoleic acids and traces of heptadeceneoic and stearic acids accounting for the rest. (Figure 5. 2).

In all cases of cells grown on even-numbered n-alkanes, from n-decane to n-hexadecane, the ratio of oleic to linoleic acids was about 3:1. These were again the major fatty acid constituents of the cell with small amounts of the other fatty acids also present, (Figure 5. 3, Table 5. 2). There were no significant changes in the cellular fatty acid profiles for S. lipolytica at different growth phases in aerobic batch culture on any substrate tested under the culture conditions described.

S. lipolytica cells grown on odd-numbered n-alkanes showed a progressive incorporation of heptadeceneoic ($C_{17:1}$) acid in going from n-undecane, n-tri-decane to n-pentadecane; with a corresponding decrease in the amounts of oleic and linoleic

Figure 5.1



Gas-liquid chromatograph of methyl esters of the fatty acids present in S. cerevisiae, strain D22 cells, after growth on 0.5% (v/v) ethanol to stationary phase.

TABLE 5.1.

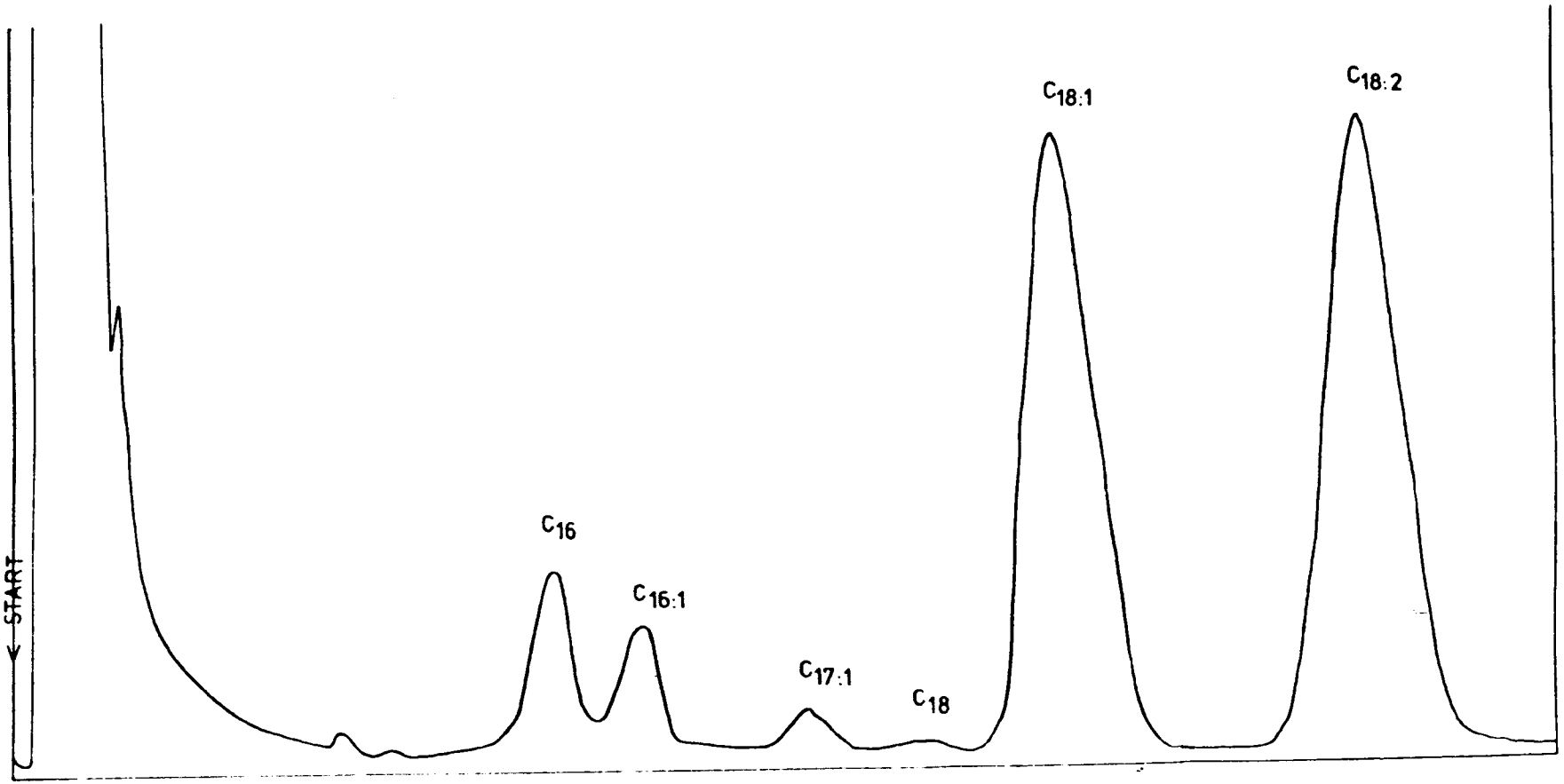
Fatty acid composition of S. cerevisiae cells.

Strain	Type	Class	C_{15}	C_{16}	C_{17}	C_{18}		$C_{16:1}$	$C_{17:1}$	$C_{18:1}$		Total % UFA
D22	wild type	-	-	11	-	6		47	-	36		83
D22-DCS12	$TTFB^R$	2	-	10	-	6		44	-	40		84
D22-CB19	"1799"^{R}	2	-	11	-	5		43	-	41		84
D22-EC1	TET^R	2	-	7	-	4		53	-	36		89

Notes.

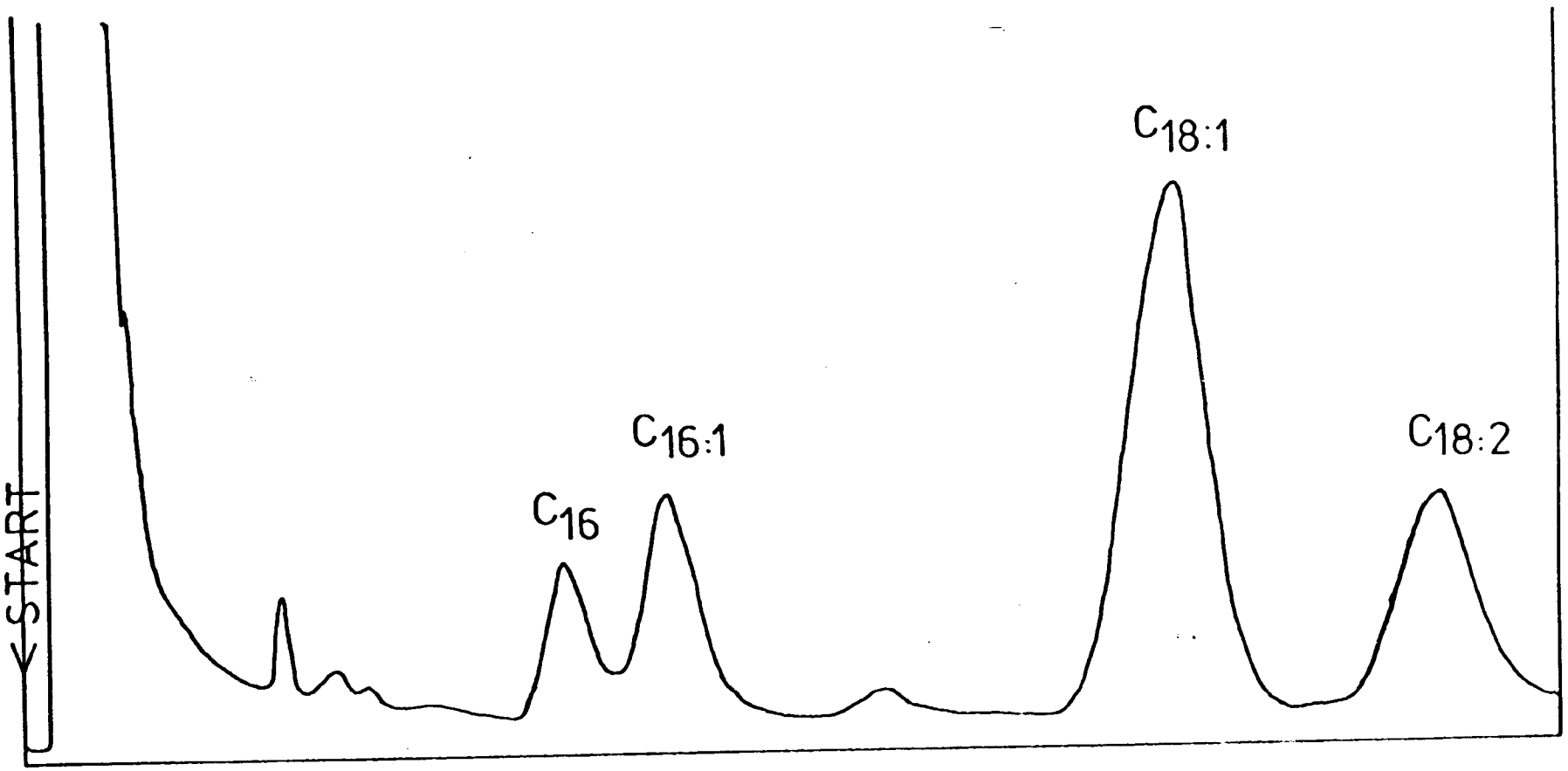
1. Fatty acids are shown as % of total fatty acids and denoted by the convention, number of carbon atoms: number of double bonds.
2. UFA = unsaturated fatty acids.
3. All cells grown on ethanol and harvested in stationary phase.

Figure 5.2



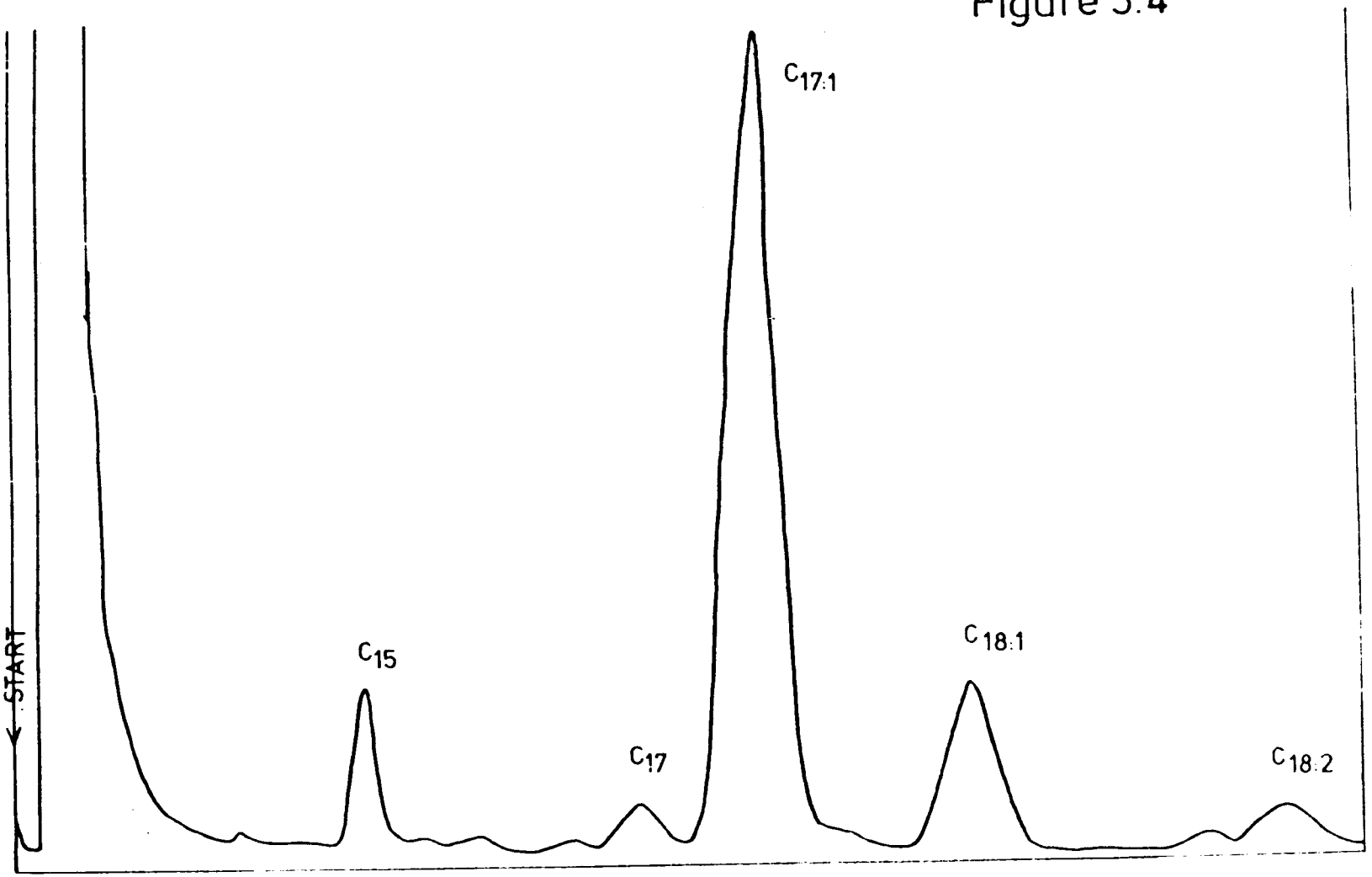
Gas-liquid chromatograph of methyl esters of the fatty acids present in S. lipolytica cells grown on ethanol (0.5% v/v) to stationary phase.

Figure 5.3



Gas-liquid chromatograph of methyl esters of the fatty acids present in S. lipolytica cells grown on n-tetradecane to stationary phase.

Figure 5.4



Gas-liquid chromatograph of methyl esters of the fatty acids present in S. lipolytica cells grown on n-pentadecane to stationary phase.

TABLE 5.2.

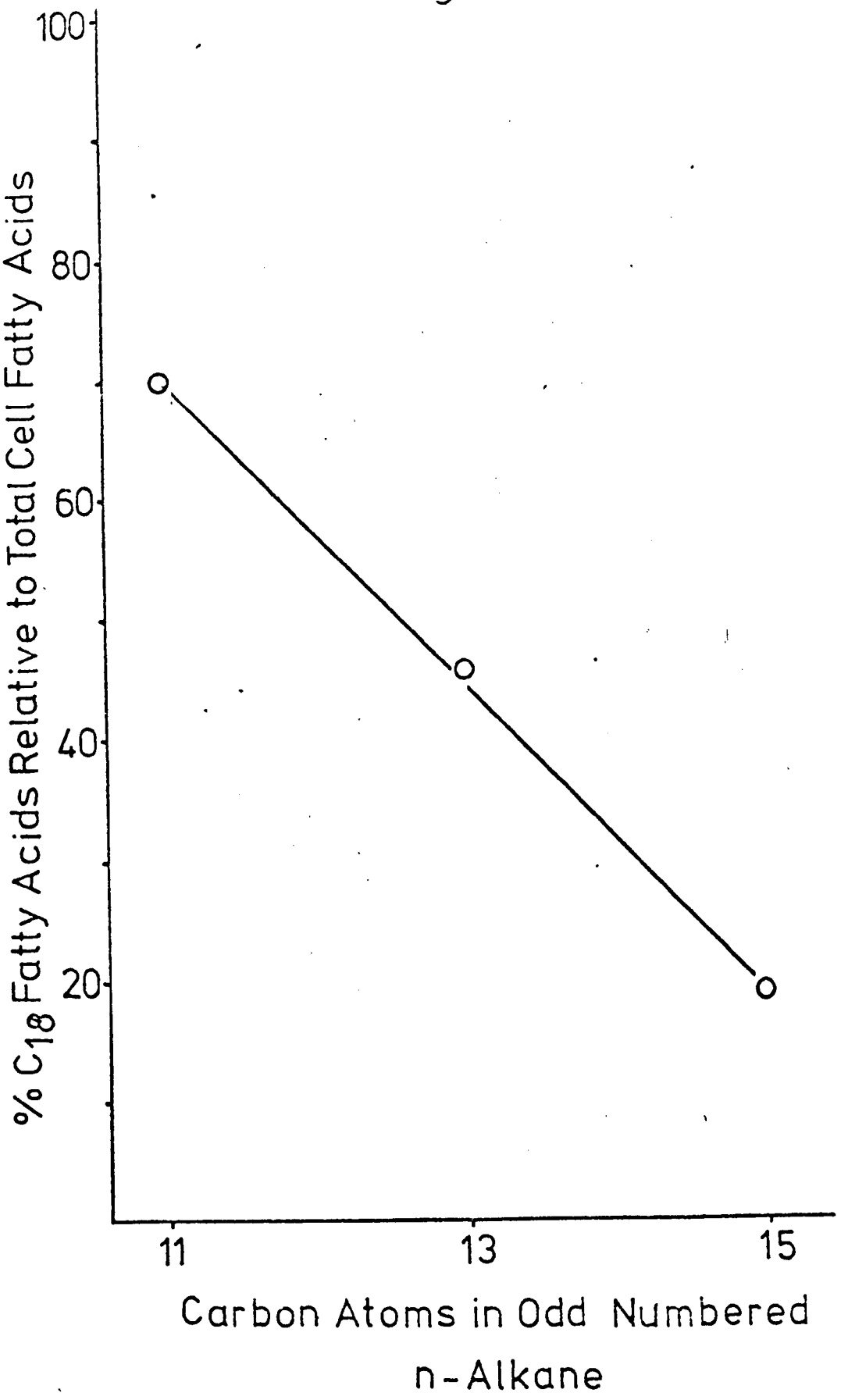
Fatty acid composition of S. lipolytica and S. cerevisiae cells grown on different substrates.

Substrate	$<C_{15}$	C_{15}	C_{16}	C_{17}	C_{18}	$C_{16:1}$	$C_{17:1}$	$C_{18:1}$	$C_{18:2}$	Total % UFA
S. lipolytica										
Ethanol	-	-	7	1	1	6	6	38	41	91
Glucose (Log)	3	-	11	-	2	5	2	39	38	84
n-Decane	-	-	8	-	-	3	2	64	23	92
n-Dodecane	-	-	9	-	2	9	3	61	15	89
n-Tetradecane	3	-	7	-	-	9	4	57	18	88
n-Hexadecane	-	-	7	-	-	9	2	68	14	92
n-Undecane	2	-	9	-	-	8	11	60	10	89
n-Tridecane	- -	8	4	3	2	5	32	39	7	83
n-Pentadecane	-	5	1	3	-	-	72	12	7	91
S. cerevisiae (strain D22)										
Glucose (Log)	3	-	13	-	5	44	-	36	-	80

Notes.

1. Fatty acids are shown as % of total fatty acids and denoted by the convention, number of carbon atoms: number of double bonds.
2. UFA = unsaturated fatty acids.
3. All cells harvested in stationary phase unless otherwise specified.

Figure 5 5



Percentage of fatty acids with 18 carbon atoms in chain length, present in S. lipolytica cells grown to stationary phase on odd-numbered n-alkanes.

TABLE 5.3.

Fatty acid composition of S. lipolytica and S. cerevisiae mitochondria isolated from cells grown on different substrates.

Substrate	$<C_{15}$	C_{15}	C_{16}	C_{17}	C_{18}	$C_{16:1}$	$C_{17:1}$	$C_{18:1}$	$C_{18:2}$	Total % UFA
S. lipolytica										
Ethanol	2	-	9	1	1	9	1	40	37	87
n-Tetradecane	1	-	9	-	2	7	3	55	22	87
n-Pentadecane	3	6	4	-	-	3	56	19	9	87
S. cerevisiae (strain D22)										
Ethanol	-	-	13	-	7	45	-	35	-	80

Notes.

1. Fatty acids are shown as % of total acids and denoted by the convention, number of carbon atoms : number of double bonds.
2. UFA = unsaturated fatty acids.

acids (Table 5.2). The fatty acid profile of cells grown to stationary phase on n-pentadecane is shown in Figure 5.4. A small amount of n-pentadeceneoic acid is also present. Only in the cases of growth of S. lipolytica to stationary phase on n-tridecane or n-pentadecane were fatty acids corresponding in chain length to the hydrocarbon substrate found in measureable amounts. There is a linear relationship between the relative amount of fatty acids with eighteen carbon atoms, present after growth on odd-numbered n-alkanes, and the number of carbon atoms in the n-paraffin (Figure 5.5). Hug and Fiechter, (1973) show a similar situation in Candida tropicalis cells. The bio-synthesis of fatty acids therefore appears to be affected by growth on odd-numbered n-alkanes.

The fatty acid composition of S. cerevisiae or S. lipolytica mitochondria was similar to the corresponding intact cells and could also be varied by growth on odd-numbered n-paraffins (Table 5.3). The large difference in fatty acid profile between S. cerevisiae and S. lipolytica raises the possibility of variations in the properties of the mitochondria from these two species of yeast. While the biochemical characteristics of S. cerevisiae mitochondria have been extensively documented (Ohnishi et al, 1966a; Ohnishi et al, 1967; Kovac et al, 1968; Mackler and Haynes, 1973), there have been few reports on Candida lipolytica or S. lipolytica mitochondria, particularly in the case of cells grown on hydrocarbons.

The effect of temperature on the activities of mitochondrial, membrane bound enzymes in S. cerevisiae and S. lipolytica was examined. These experiments were prompted by previous reports which indicate that phase transition temperatures in mitochondria are influenced by the fatty acid constitutents of the inner membrane (Ainsworth et al, 1972; Watson et al, 1973). Figure 5.6 shows Arrhenius plots for NADH and succinate oxidases in mitochondria isolated from ethanol grown, early stationary phase cells of S. cerevisiae. Transition temperatures for both these membrane bound enzymes were in the range 14-17^{o}C. At values below this temperature there was an increase in activation energy. For mitochondria isolated from S. lipolytica cells grown under the same conditions, the corresponding enzymes showed transition temperatures in the range 14-17^{o}C (Figure 5.7). These observations were made over 3-4 separate mitochondrial preparations from each species of yeast. The specific activities of the NADH and succinate oxidases in S. lipolytica were at least twofold higher than in S. cerevisiae mitochondria (Table 5.4). However, the NADH oxidase in all cases had a higher specific activity than the corresponding succinate oxidase and was also characterised

FIGURE 5.6

Arrhenius plots of respiratory enzymes in S. cerevisiae, strain D22 mitochondria. The cells were grown to stationary phase on ethanol (0.5% v/v). Mitochondria were isolated using the Braun shaker and gradient purified before use.

Figure 5.6

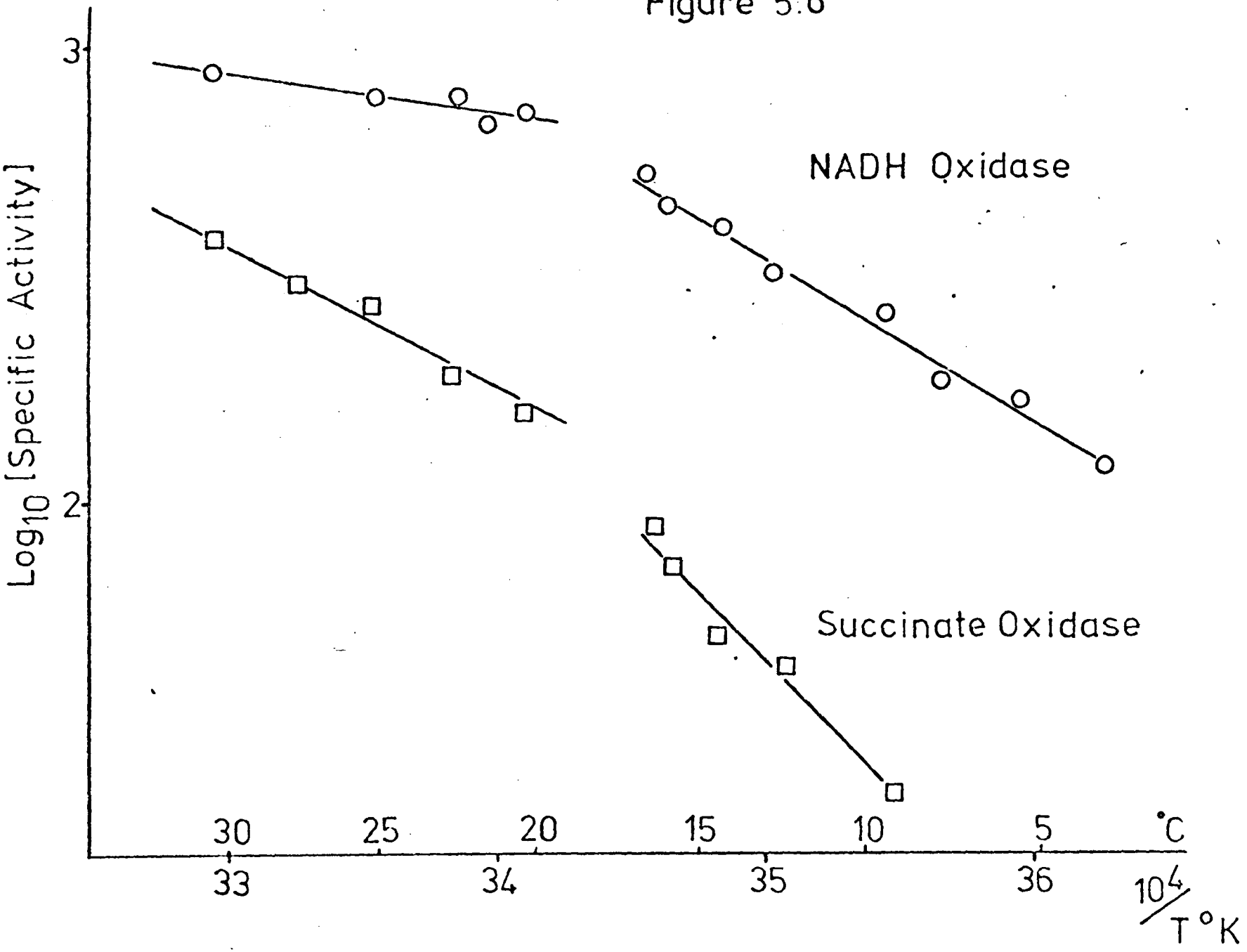

3
2
Log10 [Specific Activity]
NADH Oxidase
Succinate Oxidase
30
25
20
15
10
5
°C
33
34
35
36
10^4/T °K

FIGURE 5.7

Arrhenius plots of respiratory enzymes in S. lipolytica mitochondria. The cells were grown to stationary phase on ethanol (0.5% v/v). Mitochondria were isolated using the Braun shaker and gradient purified before use.

Figure 5.7

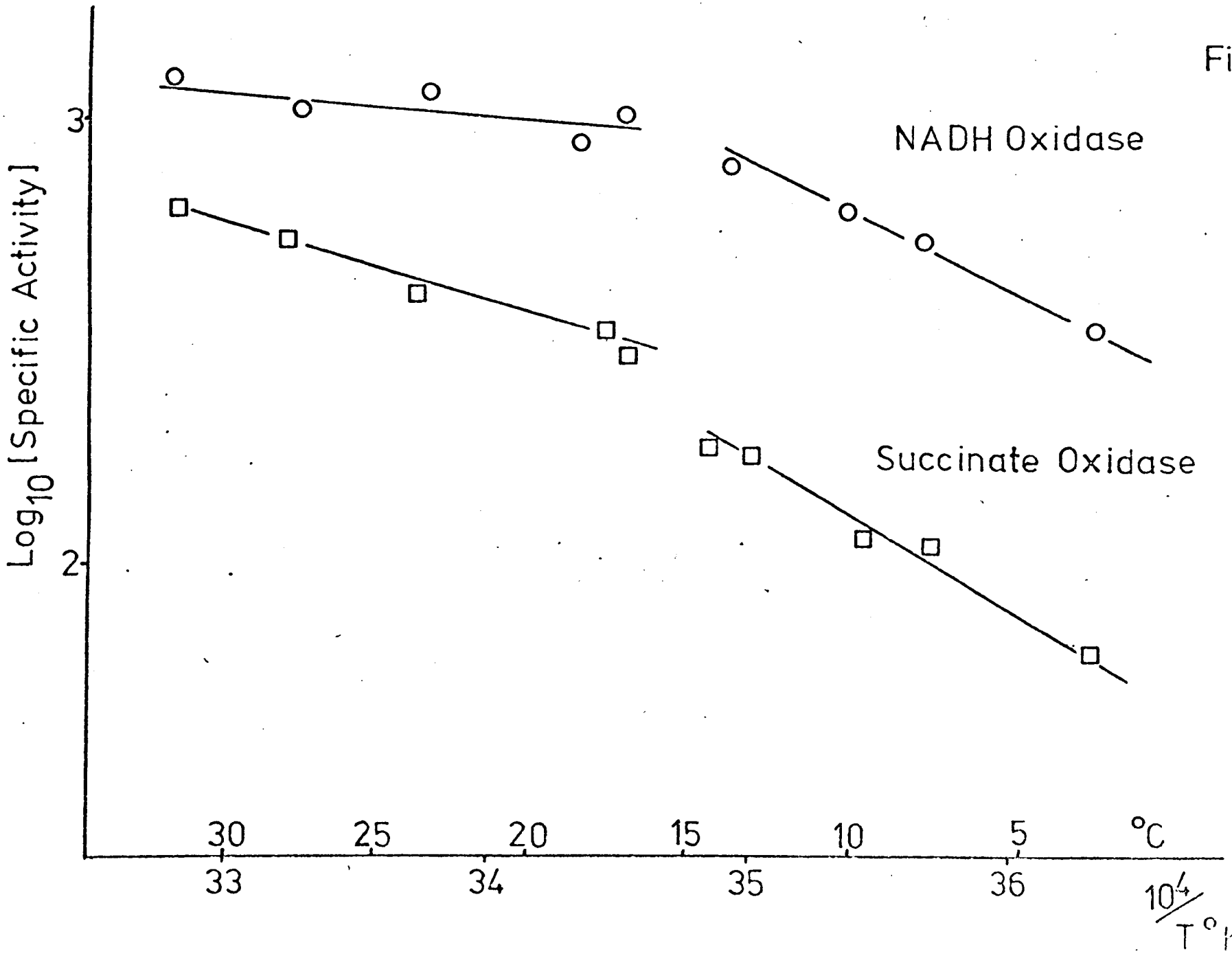
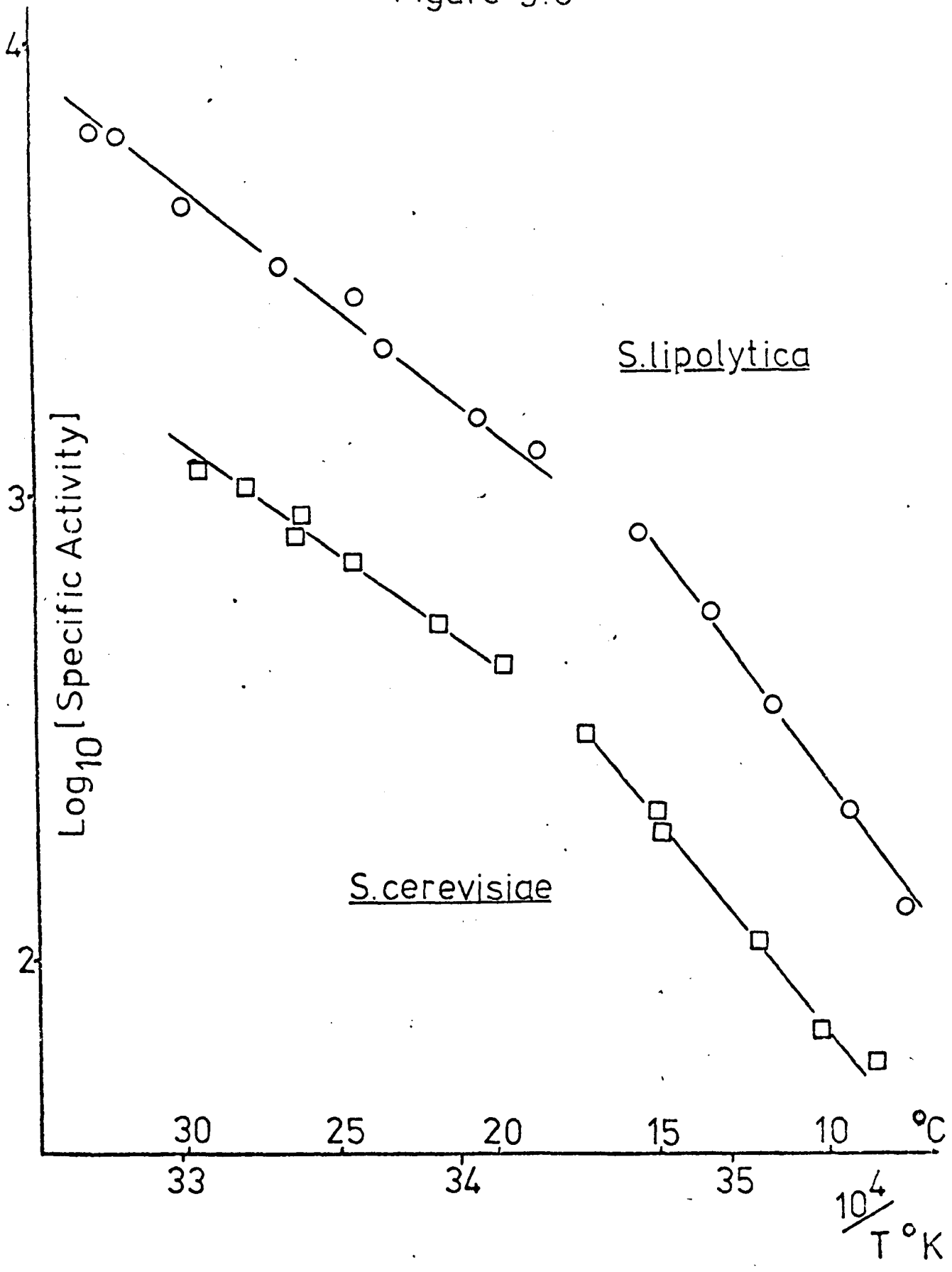

NADH Oxidase
Succinate Oxidase
Log10 [Specific Activity]
3
2
30
25
20
15
10
5
°C
33
34
35
36
10⁴/T °K

Figure 5.8



Arrhenius plots of the succinate dehydrogenase activities present in S. cerevisiae, strain D22, and S. lipolytica mitochondria. These were prepared from ethanol grown cells as described in Figures 5.6 and 5.7 respectively.

FIGURE 5.9

Arrhenius plots of respiratory enzymes in S. lipolytica mitochondria. The cells were grown to stationary phase on n-tetradecane (0.2% v/v). Mitochondria were prepared using the Braun shaker and gradient purified before use.

O - O NADH Oxidase.

□ - □ Succinate Oxidase.

Δ - Δ Succinate Dehydrogenase.

Figure 5.9

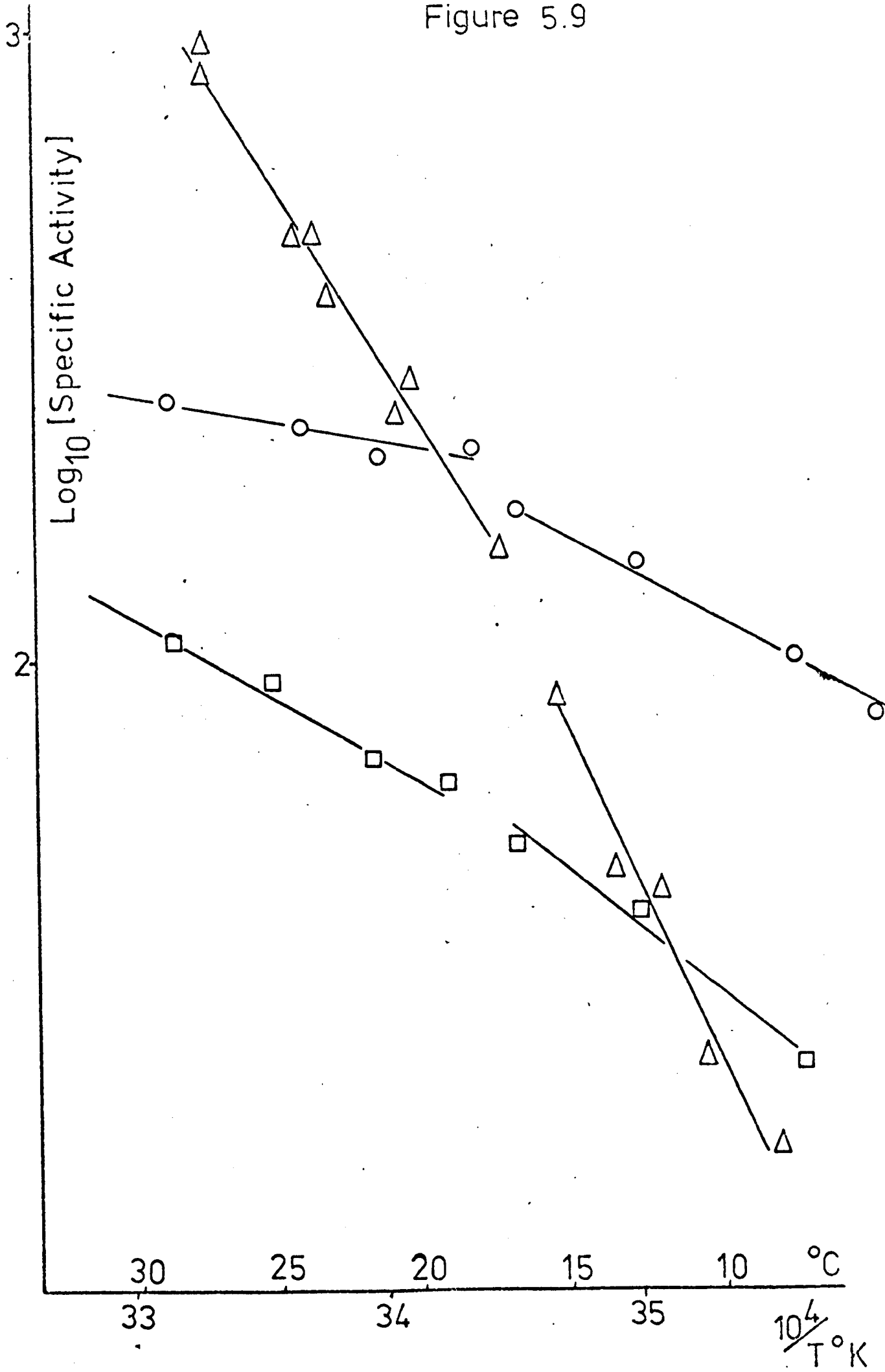


TABLE 5.4.

Transition temperatures and activation energies of S. lipolytica and S. cerevisiae mitochondrial membrane-bound enzymes.

Growth Substrate	Enzyme	Specific Activity nmole substrate/min/ mg protein	Transition Temperature °C	Activation Energy Kcal/mole	
				Above Transition	Below Transition
S. lipolytica					
Ethanol	NADH Oxidase	2140	14-17	2.2	8.4
	Succinate Oxidase	980	14-17	8.2	16.5
	[1]SDH	4000	14-17	21.2	36.9
n-Tetradecane	NADH Oxidase	520	17-20	3.5	10.1
	Succinate Oxidase	230	17-20	10.4	15.2
	[1]SDH	1300	15-18	30.2	38.3
S. cerevisiae (strain D22)					
Ethanol	NADH Oxidase	800	17-20	6.2	20.4
	Succinate Oxidase	410	17-20	14.7	23.9
	[1]SDH	1100	17-20	20.1	37.8

[1]SDH = succinate dehydrogenase.

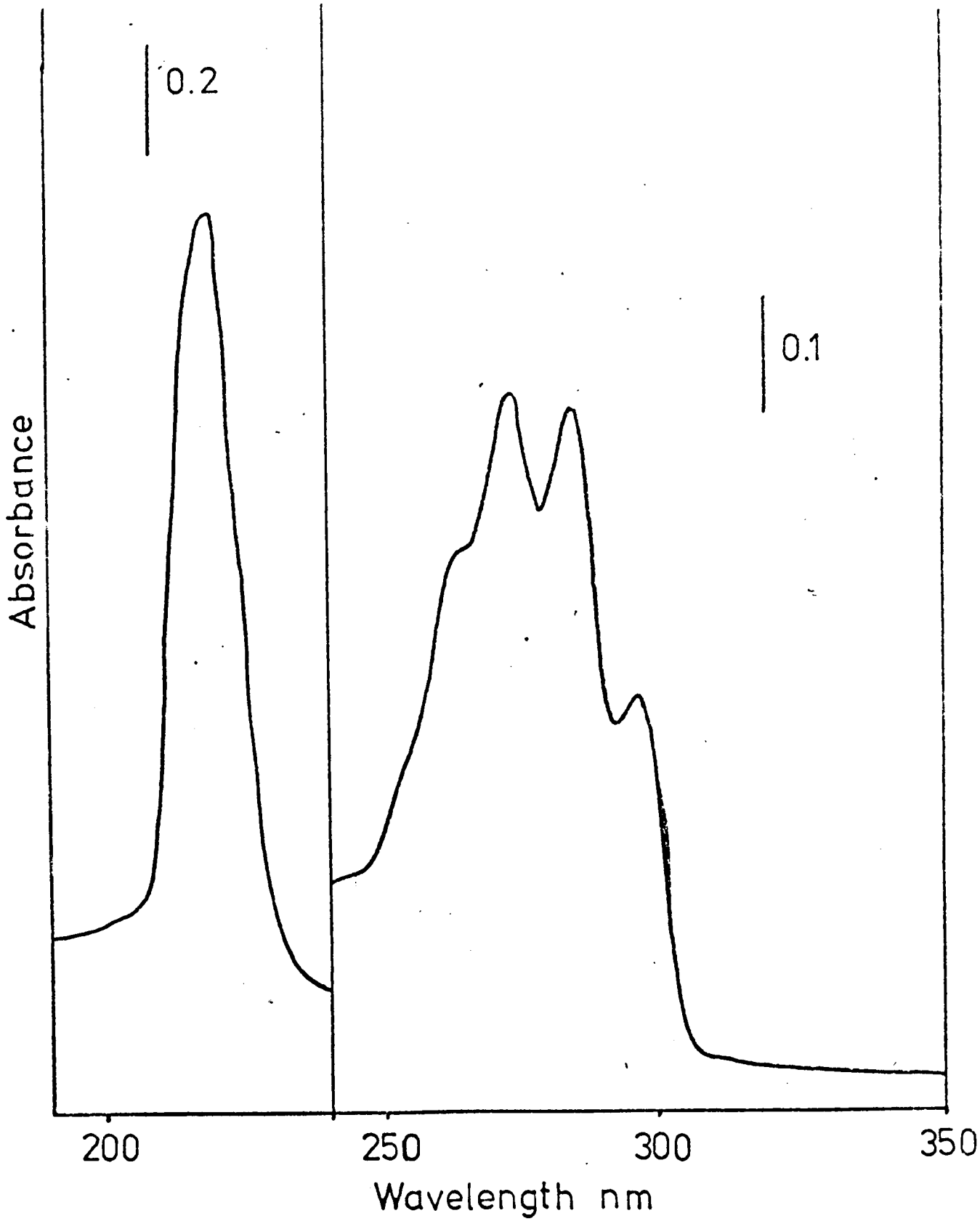
by a lower activation energy, especially above the transition temperature (Table 5.4). Succinate oxidase activities decreased rapidly at temperatures less than the transition value (Figures 5.6, 5.7). The activation energies, both above and below the transition temperature, for the S. cerevisiae NADH and succinate oxidases were higher than those of the corresponding enzymes in S. lipolytica mitochondria.

The mitochondrial succinate dehydrogenases also showed discontinuities in their respective Arrhenius plots (Figure 5.8). Again, the specific activity of the S. lipolytica enzyme was much higher than that in S. cerevisiae mitochondria. The transition temperatures were similar to the membrane bound oxidases. However, in contrast to these enzymes, the activation energies of succinate dehydrogenases, both above and below the transition temperatures, were in the same range for both species of yeast. (Table 5.4).

Arrhenius plots of membrane bound enzymes in mitochondria isolated from S. lipolytica cells grown to stationary phase on n-tetradecane are shown in Figure 5.9. Each has a lower specific activity and a higher transition temperature relative to the corresponding enzyme in ethanol grown cells (Table 5.4). The activation energies for these enzymes were also greater than those measured in S. lipolytica grown on ethanol (Table 5.4). The activation energy of the succinate dehydrogenase was especially high. However, this observation may be due to an artefact of the isolation procedure, since mitochondria from alkane grown cells were produced only by the Braun shaker method. More intact mitochondria could be isolated using the snail enzyme procedure, but this was only available for ethanol grown cells.

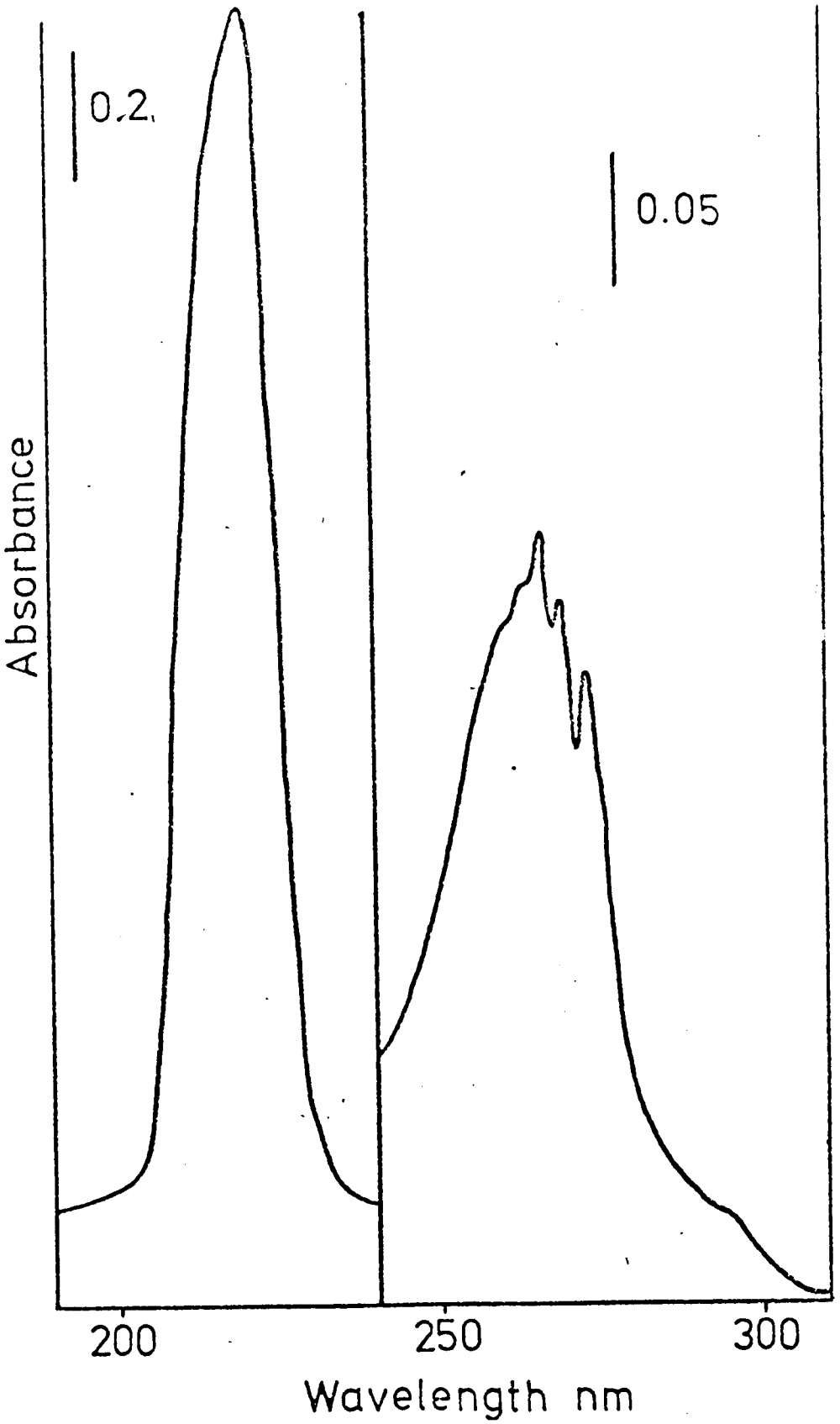
Sterols have been assayed qualitatively in S. cerevisiae, strain D22 cells grown on ethanol (Figure 5.10). There are absorption peaks at 293 nm, 281 nm and 271 nm with a shoulder at 262 nm. This spectrum is attributed to ergosterol and 24,(28)-dehydroergosterol. In addition, 24,(28)-dehydroergosterol has a peak between 220 nm and 230 nm which is not present in ergosterol. The intense absorption between 200 nm and 240 nm in Figure 5.10 is assymmetric and may be due to contributions from 24,(28)-dehydroergosterol and to poly-unsaturated compounds eg. squalene, which may also be present. The sterol contents of the uncoupler resistant mutants D22-DCS12, and D22-CB19 and of the TET^R mutant D22-EC1 have been assayed qualitatively and no differences found between these and the wild type. These results may be contrasted with those reported for other antibiotic resistant strains (Woods, 1971; Molzahn and Woods, 1972). Qualitative representation of the sterol content of S. lipolytica cells grown on ethanol is shown by the absorption

Figure 5.10



Absorption spectrum of sterol extract from S. cerevisiae, strain D22, grown to stationary phase on ethanol (0.5% v/v).

Figure 5.11



Absorption spectrum of sterol extract from S. lipolytica grown to stationary phase on ethanol (0.5% v/v).

spectrum in Figure 5.11. There are maxima at 266 nm, 269 nm and 273 nm together with a large asymmetric peak at 220 nm. The sterols present have not been identified. When the cells were grown on any n-alkane from n-decane to n-hexadecane the absorption between 240 nm and 300 nm decreased relative to that at 200 nm.

DISCUSSION

The fatty acid profiles of intact cells of S. cerevisiae (Table 5. 1) compare with the results of Watson et al, (1971). No significant differences were found between the wild type and any of the mutants and there was no evidence for the presence of any unusual fatty acids. The fatty acid constitutents of the mitochondria were essentially the same as the corresponding intact cells (Table 5. 3).

Characteristic structural features of the mitochondrion are the double membrane and well defined cristae (Palade, 1953). In yeast mitochondria the phospholipids present are similar to those in mammalian species (Jakovic et al, 1971). Phosphatidyl choline and phosphatidyl ethanolamine make up over 70% of the total, with small amounts of other types also present. Cardiolipin, localised exclusively in the inner membrane accounts for 20% (McMurray and Dawson, 1969).

The inner mitochondrial membrane has a relatively high protein: lipid ratio (Racker, 1970; Guidotti, 1972; Kagawa, 1972) and the fatty acid components exhibit a relatively high degree of unsaturation together with a very low sterol : phospholipid ratio (0. 05 :1). Within these constraints there is great variability in fatty acid distribution. Along with species differences, environmental factors may also affect the relative fatty acid profile (Chapman and Leslie, 1970; Watson et al, 1971).

The major fatty acids present in S. lipolytica after growth on various substrates were very different from those characteristic of S. cerevisiae (Table 5. 2). The lipid composition of the cells may also depend on the temperature of growth, the extent of aeration and the growth phase. Especially in the case of growth on n-alkanes it is necessary to distinguish between those fatty acids present as metabolites and those which are components of membrane phospholipids. Glucose or ethanol grown cells of S. lipolytica have approximately equal amounts of the unsaturated $C_{18:1}$ (oleic) and $C_{18:2}$(linoleic) acids as their major fatty acid constituents. These were also the major fatty acid components when the cells were grown on n-alkanes with an even number of carbon atoms (Table 5. 2)although $C_{18:1}$ predominated. Chain elongation and desaturation must occur, even if it is presumed that fatty acids which are the same carbon chain length as the n-paraffin arise directly from the initial

hydrocarbon oxidation (Klug and Markovetz, 1971; Gallo et al, 1973 a, b). There may be a cellular requirement for oleic and linoleic acids because they are present even after growth on n-alkanes with an odd number of carbon atoms.

The yeast Candida lipolytica may exhibit changes in fatty acid profile during growth in batch culture on n-paraffins (Kates and Baxter, 1962). Similar results have also been obtained by Hug and Fiechter, (1973) for C. tropicalis. However these authors used relatively high concentrations of hydrocarbon ($\geq$ 1.0% w/v) and report a predominance of fatty acid with carbon chain length corresponding to that of the n-alkane substrate in each case. For S. lipolytica grown on low concentrations of n-paraffins (0.1-0.4% v/v) the predominant fatty acids in the cells were of longer chain length than the substrate in every case (Table 5.2). In this case the fatty acid profile of the cells was reflected in that of isolated mitochondria (Table 5.3). It is feasible therefore, that the relatively large amount of fatty acid with the same chain length as the n-alkane substrate, observed by Hug and Fiechter, (1973), may be present only as the initial product of n-alkane oxidation and not as a membrane component.

Klug and Markovetz, (1965) find that, after batch culture of C. lipolytica on n-alkanes, the fatty acids present in the culture medium are mostly of the same carbon chain length as the substrate. Relatively high concentrations of n-paraffins were used and these fatty acids may have been excreted as products of the initial hydrocarbon oxidation. The phospholipid components of C. lipolytica grown either on glucose or on n-alkane may be compared (Chenouda and Jwanny, 1972). Although there are no differences the alkane grown cells contain larger amounts of stored triglycerides (Thorpe and Ratledge, 1972).

Several external factors may affect the fatty acids of yeast grown on n-alkanes. The degree of unsaturation of the lipids in the cell is higher at lower temperatures (Kates and Paradis, 1973), and this would also affect membrane fluidity. At low rates of aeration, fatty acids directly derived from the oxidation of n-paraffins are (with elongation and desaturation) directly incorporated into the cell structure. At higher rates, these are broken down to smaller fragments which are used to resynthesise the longer chain fatty acids.

During growth of S. lipolytica on n-alkanes with an odd number of carbon atoms the stearic, oleic and linoleic acids which are present may arise either by de novo synthesis or by α-oxidation with subsequent chain elongation. The content of fatty acids with carbon chain length C_{18} is therefore a measure of fatty acid biosynthesis (Figure 5.5). Long chain n-alkanes and/or the fatty acids directly

obtained from them inhibit de novo synthesis. Growth of Candida sp. on n-paraffins will repress the fatty acid synthetase complex (Gill and Ratledge, 1973).

The predominant fatty acids in S. lipolytica are oleic and linoeic when the yeast are grown aerobically on ethanol, glucose or even-numbered n-alkanes. Growth on n-paraffins causes an increase in the amount of oleic acid, relative to linoleic. A single double bond in the largest fatty acid component was also preferred when the yeast were grown on odd-numbered n-alkanes. Glucose or ethanol grown cells are therefore relatively highly unsaturated, having the largest contents of linoleic acid ($C_{18:2}$). The presence of only a single double bond in the major fatty acid constituent of S. lipolytica grown on n-alkanes may contribute to the maintainence of membrane integrity, in the presence of such lipophilic substrates. Differences in cell membrane structure occur between cells grown on hydrocarbons or on glucose (Ludvik et al, 1968).

Biological membranes are stabilised mainly by electrostatic and hydrophobic interactions. In addition, hydrated phospholipids exhibit lyotropic and thermotropic mesomorphism. Electrostatic interactions between protein (cytochrome c) and phospholipid dispersions can result in the formation of water-soluble complexes (Reich and Wainio, 1961; Gulik-Krzywicki et al, 1969; Kimelberg et al, 1970). After initial ionic binding the cytochrome c may penetrate to the interior of the phospholipid lamellae with concomitant hydrophobic interactions (Das and Crane, 1964; Shipley et al, 1969; Kimelberg and Papahadjopoulos, 1971 a, b).

Apart from studies on model systems, several hypotheses of membrane structure have been advanced from experiments on isolated membranes. The original concept of the lipid bilayer (Gorter and Grendel, 1925), modified by Danielli and Davson, (1935) led to the formulation of the unit membrane hypothesis (Robertson, 1966). This assumes that all membranes are constructed from the same basic unit, and as such it has come under criticism (Korn, 1966; Vandenheuvel, 1971). Neither do these classical models take account of hydrophobic interactions. Such bonding was initially considered by Kauzmann, (1959) in relation to protein structure. Hydrophobic forces between proteins and phospholipids in mitochondrial membranes also exist (Richardson et al, 1963; Green and Fleischer, 1963). This may not be conclusive argument for the incorporation of proteins into the interior of a lipid bilayer, but Green and Perdue, (1966) suggested that biological membranes were made up of such lipid-protein subunits.

Benson et al, (1971) have made the distinction between the class of membranes represented by myelin with lipid bilayer structures; and that including the inner mitochondrial membrane, which are made up of lipoprotein subunits. Sjostrand, (1963, 1970) considers specific lipoprotein complexes to be present in the mitochondrial membrane. In contrast, Glaser and co-workers (Glaser et al, 1970; Glaser and Singer, 1971) propose that mitochondrial proteins are randomly dispersed in the lipid layer. Although these hypotheses incorporate hydrophobic interactions they fail to account for the bilayer structure present in mitochondrial membranes.

A double layer of globular protein molecules may form the mitochondrial membrane continuum with phospholipids in a bilayer arrangement in the spaces between (Vanderkooi and Green, 1970). This formulation is compatible with X-ray data (Vanderkooi and Sundaralingam, 1971). There may also be specific lipid-protein interactions.

Information on the properties of biological membranes has been obtained through the study of membrane-bound enzymes (Lenaz, 1972). Dynamic membranes such as the mitochondrion possess a characteristic degree of fluidity. A transition temperature exists for each particular phospholipid, below which it is in a crystalline form and above which it is in a liquid-crystal phase. For the same head group and extent of hydration, phospholipids with the more unsaturated fatty acids have lower transition temperatures (Oldfield and Chapman, 1972). These properties are modified on mixing different phospholipids ; at least four phases can be distinguished in a mitochondrial lipid extract when the temperature is varied (Gulik-Krzywicki et al, (1967). In biological membranes the presence of proteins and/or sterols will also contribute to the resultant membrane fluidity (Steim et al, 1969; Esfahani et al, 1971). Mitochondrial lipids are very unsaturated, with a relatively low sterol component, and a low transition temperature as a result. In poikilothermic organisms the relative proportion of unsaturated fatty acids increases, in order to maintain membrane fluidity at low temperatures (Ingraham, 1962; Kates and Paradis, 1973; Richardson et al, 1961, 1962).

The presence of phospholipids is usually necessary for the expression of optimal activity of mitochondrial membrane bound enzymes (Fleischer et al, 1962). In the case of those catalysing electron transport and ATP synthesis phospholipids must be present for any significant activity (Brierley et al, 1962 a, b; Bruni and Racker, 1968; Kagawa, 1972; Coleman, 1973).

Mitochondria from a homeothermic animal or a chilling sensitive plant have discontinuities in Arrhenius plots of their respiratory enzymes. In contrast, similar

plots of chilling resistant plant or a poikilothermic animal exhibit no break (Lyons and Raison, 1970 a, b; Raison and Lyons, 1971). The variation in activation energy may be the result of a conformation change of the enzyme protein(s) associated with a phase change in the lipid bilayer component of the membrane (Kumamoto et al, 1971; Raison et al, 1971 a, b). This suggestion is open to criticism in that the gross lipid phase transition may not always occur at the same temperature as the discontinuity in the Arrhenius plot (Esfahani et al, 1971; Blazyk and Steim, 1972). It may be concluded that the distribution of lipids within membranes is heterogeneous such that regions associated with enzyme proteins tend to be more fluid (de Kruyff et al, 1973). Areas of differing fluidity can exist in model membranes (Oldfield et al, 1972) and this may indicate that each enzyme protein exists in a specific micro-environment, each with a specific fluidity.

The mitochondrial NADH oxidase, succinate oxidase and succinate dehydrogenase, of S. lipolytica cells grown on ethanol show higher specific activities and lower transition temperatures, when compared to the corresponding enzymes in S. cerevisiae (Table 5.4). These increases in specific activities are supported by analyses of cytochrome spectra (Chapters 2 and 4) which indicate larger amounts of cytochromes b, and aa_3, in Saccharomycopsis mitochondria. The lower transition temperatures can be explained by the increased unsaturation of the S. lipolytica fatty acids (Table 5.3). In the n-tetradecane grown cells relative to ethanol grown there is a decrease in the specific activity of each of the enzymes (Table 5.4). This again correlates with the cytochrome content. The transition temperatures increase as the relative percentage of linoleic acid in the mitochondrial lipids decreases. The decrease in respiratory activity in n-tetradecane grown cells may be associated with the decreased overall membrane fluidity. Evidence that the S. lipolytica mitochondrial membrane may not be homogeneous in this respect comes from the succinate dehydrogenase, which is different in transition temperature from the other enzymes (Table 5.4). Other workers have observed discontinuities in Arrhenius plots of S. cerevisiae mitochondrial membrane bound enzymes and correlated them with the saturation characteristics of the lipid components (Watson et al, 1973). Although correlation of Arrhenius plots with the gross lipid composition of the mitochondrial membranes is possible, the existence of a specific environment for each mitochondrial enzyme must also be taken into account (Bertoli et al, 1973; Sechi et al, 1973). This produces difficulties in the interpretation of the relationship between lipid composition and respiratory activity in the yeast cell (Kovac et al, 1967b; Bertoli et al, 1971; Castelli et al, 1972).

The role of sterols in biological membrances is unclear. However, there are interactions between sterols, eg. cholesterol, and the hydrocarbon chains of phospholipids (Chapman and Penkett, 1966). Calorimetric studies show that cholesterol has a marked effect on the phase transitions of different phospholipids (Ladbrooke et al, 1968). Below the transition temperature, in the presence of cholesterol, the fatty acid chains are more mobile than in its absence; above the transition temperature they are less mobile. High concentrations of cholesterol produce a condition of intermediate fluidity where a non-cooperative phase transition takes place over a large temperature range (Lippert and Peticolas, 1971). In some membranes cholesterol may prevent the formation of crystalline areas, while inhibiting the motion of the fatty acid chains in more fluid regions (Ladbrooke et al, 1968; Jenkinson et al, 1969). Addition of cholesterol may lower the temperature of onset of the gel to liquid-crystalline transition. Mixed gel and liquid-crystal clusters may therefore be common in membranes which specifically lack cholesterol (Steim et al, 1969; Chapman and Urbina, 1971; Oldfield et al, 1972; de Kruyff et al, 1973). The mitochondrial lipids are relatively highly unsaturated and do not require high concentrations of sterol to maintain the liquid crystalline state; in contrast to membranes such as myelin. The low concentration of sterol in mitochondrial membranes permits the observation of a lipid phase transition.

The major sterol present in yeast is ergosterol (Breivik and Owades, 1957) together with some 24,(28)-dehydroergosterol. In S. cerevisiae, strain D22, and the various selected mutants these sterols were found (Figure 5.10). There was no evidence of any other sterol present in the mutants, in contrast to the results of Woods and co-workers (Woods, 1971; Molzahn and Woods, 1972). Chenouda and Jwanny, (1972), have shown that ergosterol is also the sterol present in largest amount in C. lipolytica cells. The sterol absorption spectrum (Figure 5.11) suggests the presence of a diene system similar to that of ergosterol. In S. cerevisiae sterols fulfill a structural function in the cell membranes (Proudlock et al, 1968). Under conditions of anaerobic growth it is possible to alter the amount of ergosterol present in the cells (Cobon and Haslam, 1973). These changes in sterol content affect the Arrhenius plot of the mitochondrial ATPase. The temperature of discontinuity decreases as the ergosterol content increases. Even the relatively small amount of sterol present in mitochondria, although not needed for maintainence of the gross membrane fluidity, may be necessary for the correct microenvironments of membrane bound enzymes.

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APPENDIX

RESEARCH CENTRE

SUNBURY-ON-THAMES

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BIOLOGICAL SCIENCES BRANCH

FILE NOTE NO. BS/FN/50

8th October, 1974

Project No. 804

RESTRICTED

THE DETECTION AND LOCALISATION OF CYTOCHROME P450 IN *Saccharomycopsis lipolytica* USING LOW TEMPERATURE (-197°C) SPECTROSCOPY

by

M. Skipton
(A BP CAPS Award Student)

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SUMMARY

The presence of a carbon monoxide binding pigment absorbing at 450 nm has been demonstrated in cells and cell-free extracts of *S. lipolytyica* grown on *n*-alkanes. On the basis of spectroscopic evidence this is thought to be cytochrome P450 and is associated with the mitochondrial fractions obtained after sucrose density gradient centrifugation of the cell fractions.

ABBREVIATIONS

NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide (Reduced)
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (Reduced)
EDTA	Ethylenediaminetetra-acetic acid
CO	Carbon Monoxide

A. INTRODUCTION

The work described in this report was carried out at the British Petroleum Research Centre, Sunbury-on-Thames, Middlesex during the industrial training period associated with a Co-operative Award in Pure Science for post-graduate study, sponsored by BP. Two months were spent working in Project 804 (New Technology Division, Biological Sciences Branch). One of the aims of this Project is the study of n-alkane oxidation by cell free extracts of the yeast Saccharomycopsis lipolytica.

Biological oxidation of n-alkanes proceeds by the attack of molecular oxygen on a terminal carbon atom. This reaction is catalysed by a hydroxylase or mono-oxygenase enzyme system according to the scheme:-

$$NADPH + H^+ + O_2 + R - CH_3 \longrightarrow NADP^+ + R - CH_2OH + H_2O$$

The alcohol is then oxidised through the aldehyde to the corresponding fatty acid which is further metabolised via β-oxidation and the citric acid cycle.

In the yeast Candida tropicalis the microsomal (3) paraffin hydroxylase system contains cytochrome P450 as the terminal oxidase (3, 4 and 20) and the components of the complete system can be isolated in soluble form (4). This has a great deal in common with the liver microsomal drug hydroxylation system, which also contains cytochrome P450 (5).

Hydroxylase systems have also been isolated from bacteria grown on n-alkanes (1), eg Pseudomonas sp (5, 6) and Corynebacterium sp (7). Cytochrome P450 may also be employed in these systems as the terminal oxidase.

The objective of this work was to use spectroscopic techniques at low temperatures to identify and locate cytochrome P450 in intact cells and in cell-free extracts of S. lipolytica grown on n-alkanes.

B. MATERIALS AND METHODS

1. Materials

Glass beads (0.25 mm diameter) were obtained from B. Braun Apparatebau, Melsungen, Germany.

Horse heart cytochrome C, NAD, NADP, NADH, NADPH were obtained from Boehringer Maunheim, GmbH, Mannheim, Germany.

The French Press was manufactured by the American Instrument Company, Silver Springs, Maryland, USA.

The Dynomill (Laboratory Type KDL) was obtained from Glen Creston, Stanmore, Middlesex, UK.

2. Experimental Methods

(a) Growth of S. lipolytica

The yeast was grown in a mineral medium (No. 809) on n-paraffins (C_{14} - C_{17}) or n-hexadecane at 30°C. The fermentation was maintained at pH 4.0 with ammonium hydroxide.

(i) Batch Culture

50 cm^3 of a stationary phase culture of S. lipolytica strain 296, grown on Sabouraud Dextrose Broth, was added to 1 litre of medium stirred at 2500 rpm. Hydrocarbon substrate was initially added to 1 g/litre with aeration at 5 - 10 litre/h. After about 5 h a further 10 - 12 g of paraffin was added and the aeration adjusted to 15 litre/h. The yield was about 1 g dry cell weight (4 g wet weight) per g n-alkane. After 24 h 40 - 50 g wet weight were usually obtained.

(ii) Continuous Culture

S. lipolytica, strain 296, was obtained from a 10 litre continuous fermenter (No. SF-3) maintained at 15 - 20 g dry weight/litre on n-paraffins (dilution rate 0.17 to 0.18 h^{-1}).

(b) Preparation of Cell Free Extracts

The yeast cells were harvested by centrifugation (4000 xg) and washed twice with distilled water. All operations were carried out at 0 - 5°C and the fractions stored at -20°C.

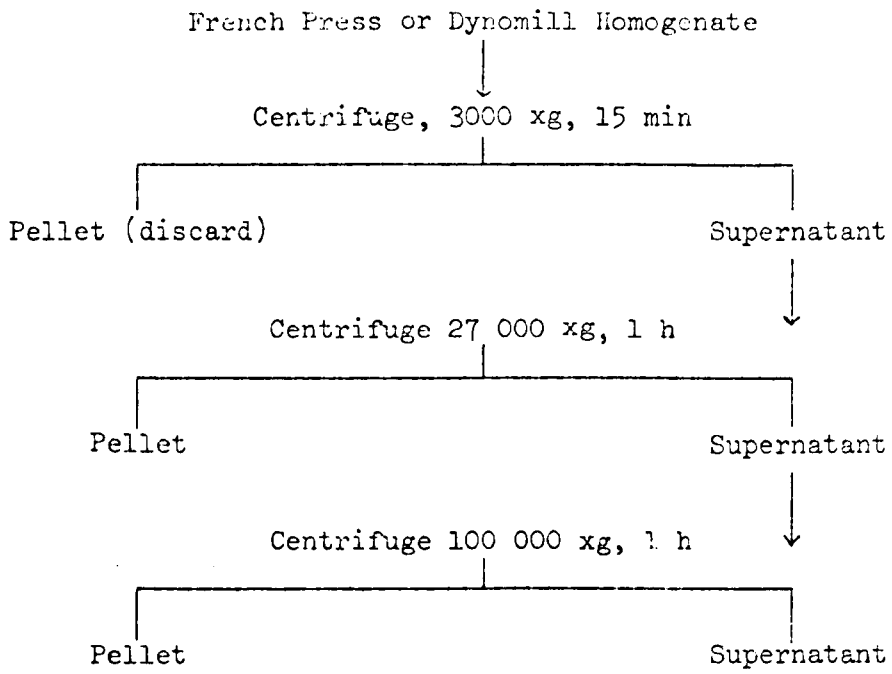
(i) Cell Disruption

Yeast cells (30 - 50 g wet weight) were resuspended (0.5 g wet weight/cm^3) in buffer containing 0.25 M Sucrose, 1 mM EDTA, 1 mM Dithicthreitol, 0.1 M potassium phosphate, pH 7.5, and passed through the French Press at 40 000 psig. This homogenate was then diluted with an equivalent volume of buffer.

Larger samples (70 g wet weight) were similarly resuspended in buffer. 150 cm^3 of suspension were placed in the 0.3 litre batch head of the Dynomill together with 250 cm^3 of glass beads (0.25 mm diameter) and the cells homogenised at 3000 rpm for 2 - 3 min. The homogenate was decanted off and the beads washed with a further 150 cm^3 of buffer, which was then added to the homogenate.

Breakage assessment was by phase contrast microscopy. Approximately 90 per cent breakage was obtained with the Dynomill and 70 per cent with the French Press.

(ii) Centrifugation



Both pellets were resuspended in buffer containing 0.25 M Sucrose, 1 mM EDTA, 1 mM Dithiothreitol, 0.1 M potassium phosphate, pH 7.5. All fractions were stored at -20°C.

(iii) Density Gradient Centrifugation

The particulate fractions were further purified by centrifugation at 100 000 xg for at least 3 h on 12 cm^3 continuous sucrose gradients (0 - 43 per cent w/v) sucrose in 0.1 M potassium phosphate, pH 7.5).

(c) Spectroscopic Techniques

Difference spectra were recorded on a Unicam SP 700C spectrophotometer at -197°C using 2 mm light path cuvettes as described by Kawai (8).

(i) Intact Cells

5 cm^3 of 0.1 M potassium phosphate, pH 7.5 was added to approximately 2 g wet weight cells. The suspension was separated into two samples. Sodium dithionite (5 - 10 mg) was added to one and the suspension left for 15 min at 0 - 5°C in order to obtain complete reduction of cytochrome P450 (9). An aliquot was added to a cuvette and immediately frozen in liquid nitrogen. Carbon monoxide was bubbled through the remainder for at least 5 min (10) and a sample frozen in a cuvette. In order to obtain an oxidised sample, 0.05 - 0.1 cm^3 of 20 vol hydrogen peroxide was added to the initial sample.

(ii) Cell Fractions

These were diluted with 0.1 M potassium phosphate, pH 7.5 to a concentration of about 8 mg/cm^3 and used instead of the intact cell suspension.

(d) Enzyme Assays

All measurements were carried out using a Perkin-Flmer 402 spectrophotometer at 30°C using 1 cm light path cells. Variations in the concentration of reduced cytochrome c at 550 nm were recorded (ϵ_{mM} = 19 (11)).

(i) Cytochrome c Oxidase

The reaction mixture (final volume 2.5 cm^3) consisted of 0.1 M potassium phosphate, pH 7.5; together with 25 - 200 µg protein and the assay was started by addition of 20 µM reduced cytochrome c.

(ii) NADH - Cytochrome c Reductase

The assay mixture (final volume 2.5 cm^3) contained 0.1 M potassium phosphate, pH 7.5; 25 - 200 µg protein; 20 µM oxidised cytochrome c; 2 mM potassium cyanide or sodium azide and the reaction was started by addition of 60 µM NADH.

(iii) NADPH - Cytochrome c Reductase

As for NADH linked enzyme except that 60 µM NADPH was used to start the assay.

(e) Protein Estimations

These were determined by the method of Lowry et al (12).

C. EXPERIMENTAL RESULTS

Experimental results are shown in Figures 1 to 6 and Tables 1 and 2.

1. Cell Fractionation

The method employed was similar to that of Gallo et al (3). The Dynomill was found to be superior to the French Press for the disruption of S. lipolytica cells. It was more efficient and the particulate fractions obtained were of better quality.

The 3000 xg pellet was seen by phase contrast microscopy to consist of cell walls and unbroken cells. The corresponding supernatant fraction was cell free.

Mitochondria were sedimented at 27 000 xg and much more compact pellets were found in the case of Dynomill homogenates. This is taken as evidence of less disintegration of the organelles.

A relatively large amount of free lipid material is present in S. lipolytica grown on n-alkanes. Fractions obtained using the French Press contain more free lipid than similar preparations using the Dynomill. This may be due to liberation of membrane lipids and, in order to absorb some of this material, bovine serum albumin (0.2 per cent w/v) was added to the cell suspension before homogenisation in the French Press.

The 100 000 xg pellet contains submitochondrial particles (produced by fragmentation of mitochondrial membranes) and the microsomal fraction of the cell. This is not homogeneous and consists of membranous vesicles, ribosomes, etc. This pellet was separated from a clear, orange-brown supernatant.

Separation of the particulate components was attempted by equilibrium density centrifugation on continuous, buffered sucrose gradients. Mitochondrial membranes are normally denser than the particulate constituents of the microsomal fraction, (Figure 1). There is essentially no difference in the separation of the 27 000 xg and 100 000 xg pellets after isopycnic centrifugation except for a pellet in the "27 000 xg" gradient. The upper (microsomal) and lower (mitochondrial) bands were diffuse, which indicates a range of particle bouyant densitites in each band.

2. Identification of Cell Fractions

Cytochrome oxidase and NADH-cytochrome c reductase are characteristic mitochondrial enzymes (3, 14). NADPH-cytochrome c reductase is specific to the microsomal fraction of yeast grown on n-alkanes (3, 14). All these enzymes were present in the 3000 xg supernatant (Table 1). The highest specific activities for each enzyme were found in the 3000 xg supernatant.

The fractions obtained from gradient centrifugation experiments (Figure 1) were also assayed for these enzymes. In neither the 27 000 xg nor the 100 000 gradient was significant mitochondrial enzymic activity observed in the microsomal fraction (Table 1). However, NADPH-cytochrome C reductase activity was found in the microsomal and mitochondrial bands from each fraction.

3. Difference Spectra of Cytochromes at -197°C

The reduced + carbon monoxide vs reduced difference spectrum of intact S. lipolytica cells grown in continuous culture on n-alkanes is compared with that of early stationary phase, glucose grown cells in Figure 2. The peak at 430 nm in both spectra is due to a shift in the absorption maximum of reduced cytochrome a_3 from 443 nm on formation of the carbon monoxide complex (10). In n-alkane grown cells, an additional band at 458 nm is observed. This is due to the presence of further carbon monoxide-binding pigments in the alkane grown cells.

A maximum at 450 nm is present in the carbon monoxide difference spectra of the particulate fractions obtained by differential centrifugation of the Dynomill homogenate of S. lipolytica cells grown in continuous culture on n-alkanes (Figure 3). This absorption maximum, which is associated with membraneous components, is present in both the 27 000 xg and 100 000 xg pellet spectra and absent in the 100 000 xg supernatant spectrum.

This absorption at 450 nm is characteristic of cytochrome P450. It is absent from S. lipolytica grown on glucose and is probably induced in the presence of n-paraffins.

Difference spectra of the samples obtained after sucrose gradient centrifugation of the 27 000 xg and 100 000 xg pellets (Figure 1) are shown in Figure 4. Cytochrome a_3 may be present in the microsomal bands (absorption at 425 nm) although no characteristic mitochondrial enzymes were detected (Table 1). There was no detectable absorption at 450 nm in the

microsomal fractions. However, cytochrome P450 was observed in the mitochondrial fractions. More cytochrome P450 seems to be present in the mitochondrial fraction isolated from the 100 000 xg pellet than from the 27 000 xg pellet.

For complete analysis of the reduced + carbon monoxide vs reduced cytochrome spectrum it is necessary to record the corresponding reduced, and reduced + carbon monoxide vs oxidised difference spectra (Figure 5). The peak at 430 nm in the reduced + carbon monoxide vs oxidised spectrum is due to the cytochrome a_3 carbon monoxide compound with contributions from other cytochromes which do not combine with carbon monoxide (Table 2). Cytochromes a and P450 give rise to the absorption bands at 440 - 450 nm. The reduced vs oxidised spectrum has an absorption at 430 nm composed of cytochromes P450 and b (Table 2). Reduced cytochromes a_3 and a absorb at 445 nm.

The α bands of the cytochromes (Figure 4) confirm the presence of the various cytochromes. Accurate analysis is not possible owing to the low sensitivity of the instrument and the linear wavenumber scale.

4. Comparison of French Press and Dynomill

Only continuously grown S. lipolytica cells were broken in the Dynomill but the French Press was used to homogenise cells grown in batch culture on n-alkanes. In this case the cells were harvested in early stationary phase.

The cytochrome difference spectra of intact cells grown in batch culture on n-alkanes provided inconclusive data on the presence of cytochrome P45 They would however contain a lesser amount than the continuously growing cells.

Carbon monoxide difference spectra of cell-free extracts of S. lipolytica continuously grown on n-alkanes and broken in the French Press (Figure 6) do however show an absorption at 450 nm. The detectable cytochrome P450 was much less than in corresponding preparations from cells broken in the Dynomill (Figure 3) - possibly due to greater membrane disintegration in the French Press. Cytochrome P450 may be converted to P420 (13) under these conditions.

D. DISCUSSION

1. Cell Disruption and Centrifugation

During the preparation of cell free extracts EDTA was present in order to inhibit lipid peroxidation. Dithiothreitol helps to stabilise cytochrome P450 in yeast (4).

The results of the gradient centrifugation separations are in contrast to those of Gallo et al for C. tropicalis (3). Both the 27 000 xg and 100 000 xg pellets gave virtually identical patterns. There was therefore incomplete separation of "mitochondria" and "microsomes" by differential centrifugation during the present study with S. lipolytica. It is possible that the presence of free lipid causes the microsomal membranes to sediment with the 27 000 xg pellet.

A range of particle bouyant densities gives rise to broad protein bands on density gradients. This was especially noticeable in the case of the mitochondrial fractions and may indicate that there are "light" and "heavy" populations of "mitochondria", the heavy species being removed at 27 000 xg. However, since there was always a layer of free lipid on the surface of the density gradients after centrifugation, differential separation of lipids from membranes might also produce the range of bouyant densities encountered.

The mitochondrial fraction present in the 100 000 xg pellet does not sediment through 43 per cent w/v sucrose. This is in contrast to the results of Gallo etal (3) for C. tropicalis and may indicate a higher lipid:protein ratio in S. lipolytica membranes.

Density gradients of the particulate fractions obtained after breakage of S. lipolytica cells using the French Press showed no clear separation of the various components. This result suggests extremely severe membrane damage in these preparations.

2. Enzyme Activities in the Cell Fractions

The descriptions of the dense band from the sucrose gradient separations as mitochondrial is confirmed by the enzymic assays (Table 2). The characteristic mitochondrial specific enzymic activities are 100 to 200 fold greater in the denser fraction than in the lighter band.

The location of NADPH-cytochrome c reductase in both bands after density gradient centrifugation is surprising. This enzyme has been considered to be specifically microsomal in origin (3, 14).

The specific activities of the mitochondrial enzymes shown in Table 1 are of the same order as those quoted by Gallo et al (3, 14). In contrast to this very low microsomal levels of NADPH-cytochrome c reductase were observed. In view of the higher specific activity of this enzyme in the 3000 xg supernatant (Table 1) it is possible that a soluble component present in the 100 000 xg supernatant is necessary for the full expression of this activity in particulate fractions from S. lipolytica.

3. Cytochrome Difference Spectroscopy at -197°C

The characteristic absorption maxima of the cytochromes are shown in Table 2. At liquid nitrogen temperatures these may be shifted towards shorter wavelengths by 2 - 3 nm. No quantitiative data may be extracted from difference spectra recorded at low temperatures. The technique does, however, produce intensification (up to 30 times) of the absorption bands so that components present in small amounts and possibly obscured by absorption bands of other cytochromes may be recognised (10).

The presence of an additional carbon monoxide binding cytochrome in S. lipolytica grown on n-alkanes compared to glucose grown cells is shown in Figure 2. This absorption at 458 nm is assigned to cytochrome P450 although the reason for the shift from the characteristic 450 nm in cell free extracts is not known. It could be a function of the whole cell environment. However, the characteristic absorption band of cytochrome a_3 is observed at the expected 430 nm in both spectra.

In cell free preparations, an absorption at 450 nm is observed in the 3000 xg supernatant and 27 000 xg and 100 000 xg pellets but not in the 100 000 Xg supernatant (Figure 3). The maximum level of cytochrome P450 appears to be in the 100 000 xg pellet. Thus cytochrome P450 appears to be associated with particulate cell free fractions of S. lipolytica. The detection of cytochrome P450 is complicated by the trough at 445 nm due to reduced cytochrome a_3 in the reference cuvette. An improvement in the measurable content of cytochrome P450 may be brought about by the presence of glycerol during the preparation of cell free extracts (4).

The peak at about 430 nm in the spectra of cell free fractions is probably due to a cytochrome a_3 carbon monoxide compound. Any shift towards the blue may be due either to contibutions from other cytochromes or to the low temperatures.

Separation of the crude fractions by gradient centrifugation shows that cytochrome P450 is localised in the mitochondrial bands and is not present in the microsomal fractions (Figure 4). The greatest amount of cytochrome P450 is present in the mitochondrial fraction obtained from the 100 000 xg pellet. This may be evidence for the existence of two mitochondrial populations in S. lipolytica. Cytochrome P450 would then be characteristic of the "light" mitochondria. The S. lipolytica hydroxylase system may therefore be more similar to the adrenal cortex hydroxylase (17) than to the rat liver (5) or C. tropicalis systems (3, 4, 18, 20).

Alternatively P450 may be localised in microsomal membranes which co-purify with mitochondrial membranes in sucrose density gradients. In order to resolve this question other methods of separation of cell components must be tried.

Solubilisation and reconstitution experiments may provide a better identification of the separate components of the hydroxylase system in S. lipolytica.

E. CONCLUSIONS

1. Cytochrome P450 has been identified in intact cells and cell free extracts of S. lipolytica grown on n-alkanes by carbon monoxide difference spectroscopy at low temperatures (-197°C).

2. The absence of cytochrome P450 in S. lipolytica grown on glucose suggests that this enzyme is induced by n-alkanes.

3. Density gradient centrifugation experiments suggest that cytochrome P450 in S. lipolytica is associated with mitochondrial rather than microsomal membranes.

4. Cytochrome P450 appears to be present in larger amounts in continuously grown cells than in batch grown cells.

5. Larger amounts of cytochrome P450 are found in cell free extracts prepared using the Dynomill than in those obtained by use of the French Press.

for M. Skipton

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G. APPENDICES

Appendix 1 - Tabulated Data

TABLE 1

ENZYME ACTIVITIES IN CELL FRACTIONS OF S. lipolytica

Sample	Sucrose Gradient Fraction	Specific Activity nmol min^{-1} mg $proteins^{-1}$		
		Cytochrome c Oxidase	NADH-Cytochrome c Reductase	NADPH-Cytochrome Reductase
3000 xg Supernatant	-	280	131	23
27 000 xg Pellet	1	1.1	2.3	0
	2	1.0	1.5	1.4
	3	40	23	6.1
	4	177	106	8.3
100 000 xg Pellet	1	0	3.2	0
	2	2.0	0	2.3
	3	32	19	4.9
	4	252	98	10.8

TABLE 2

ABSORPTION MAXIMA OF CYTOCHROMES

Cytochrome	Temperature °C	Wavelength nm					Reference
		Reduced			Reduced + CO	Oxidised γ	
		α	β	γ			
a	25	605	-[a]	444	-	425	15, 13
a_3	25	605	-	444	430, 590	830	15, 13
a	-197	601	-	447, 442		426	16
a_3	-197	604	-	444		412	16
b	25	563	532	429	-	418	15
c	25	550	521	416	-	410[b]	15
c	-197	549, 546	525, 519	415	-		15
c_1	25	553	524	418	-	410	15
P450[d]	25	555	-	420	449, 555	415[c]	13

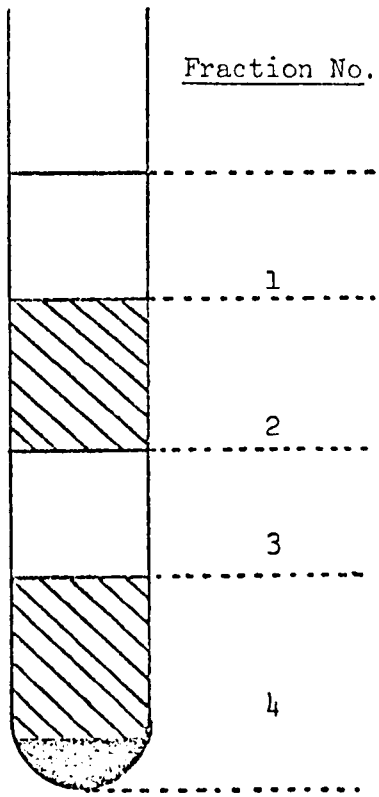
a A dash indicates no absorption, a blank indicates no data available.

b Also 529 nm.

c Also 535, 570 and 650 nm.

d Difference spectrum (reduced vs oxidised) 433 and 553 nm (peaks); 413 and 557 nm (troughs) (15).

SUCROSE GRADIENT CENTRIFUGATION OF 27 000 xg AND 100 000 xg PELLETS



Fraction No.	Protein Concentration (mg/cm^3) 27 000 xg Pellet	100 000 xg Pellet
1	1.3	0.9
2	4.8	2.4
3	1.6	1.7
4	6.5	2.5

0% (w/v) Sucrose

43% (w/v) Sucrose

Pellet (27 000 xg fraction only)

FIGURE 1

DIFFERENCE SPECTRA (REDUCED + CO vs REDUCED)
OF S. lipolytica CELLS GROWN ON n-ALKANE AND GLUCOSE

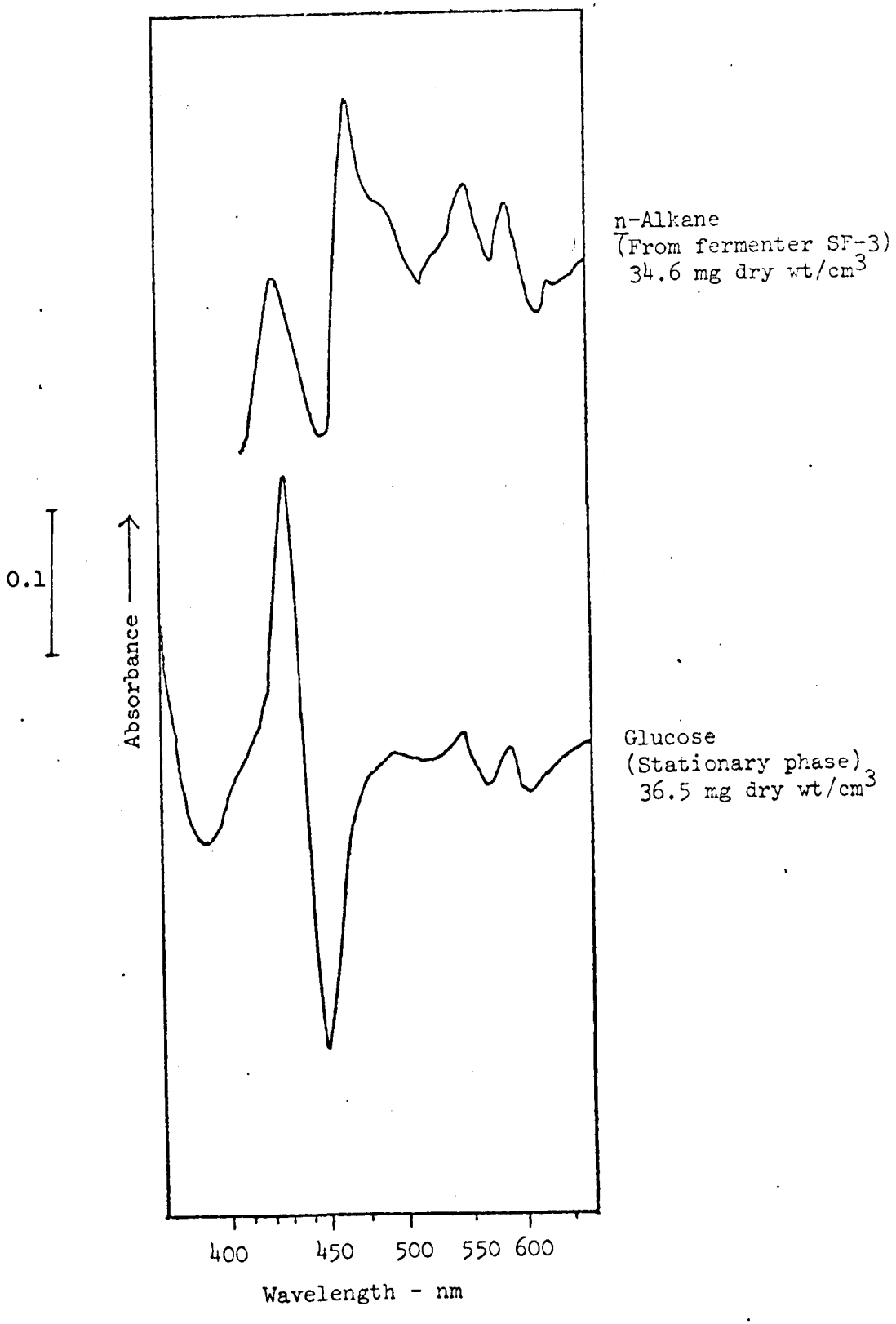


FIGURE 2

DIFFERENCE SPECTRA (REDUCED + CO vs REDUCED)
OF CELL FRACTIONS FROM S. lipolytica

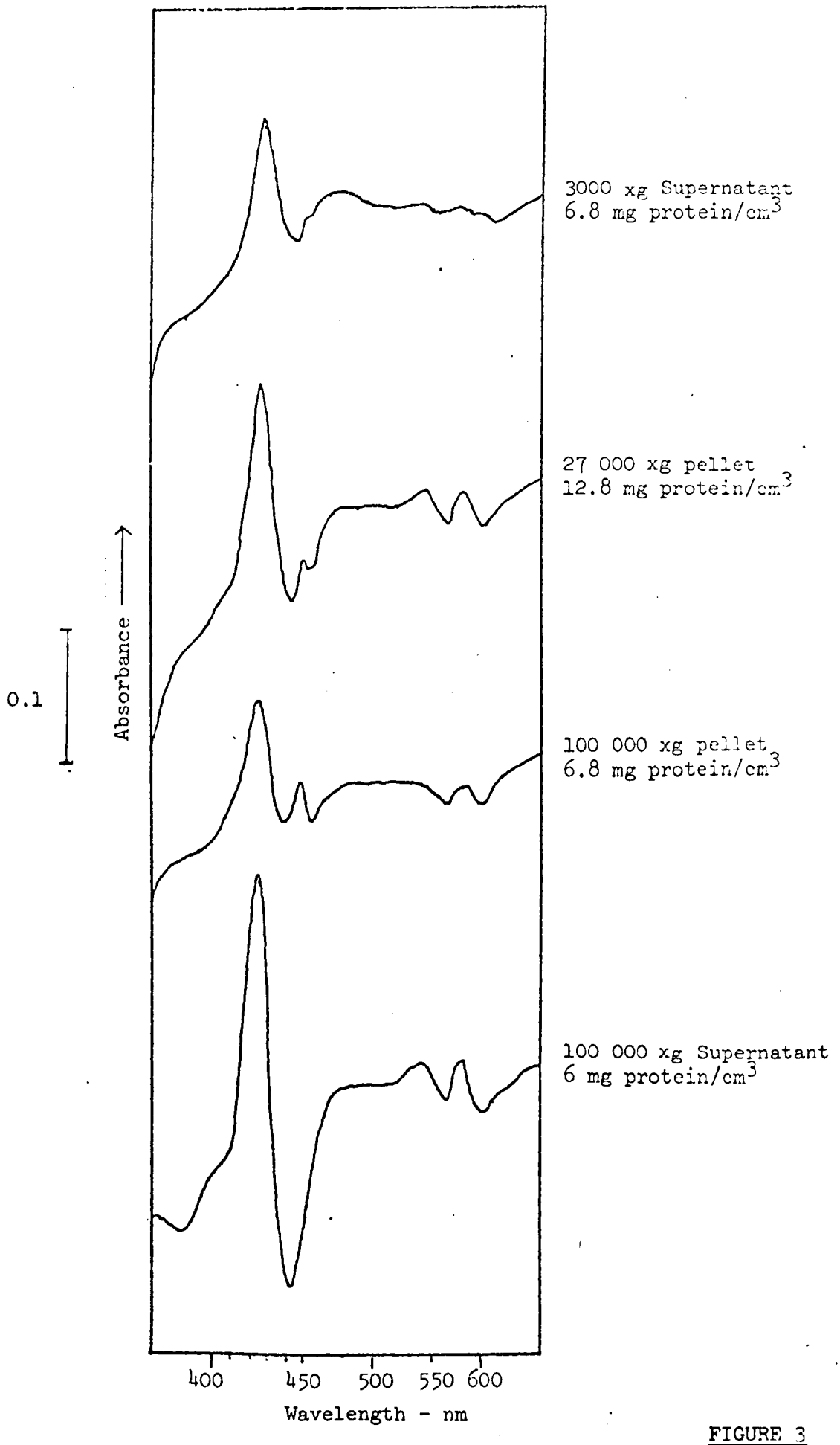


FIGURE 3

DIFFERENCE SPECTRA (REDUCED + CO vs REDUCED)
OF CELL FRACTIONS FROM S. lipolytica
AFTER SUCROSE GRADIENT CENTRIFUGATION

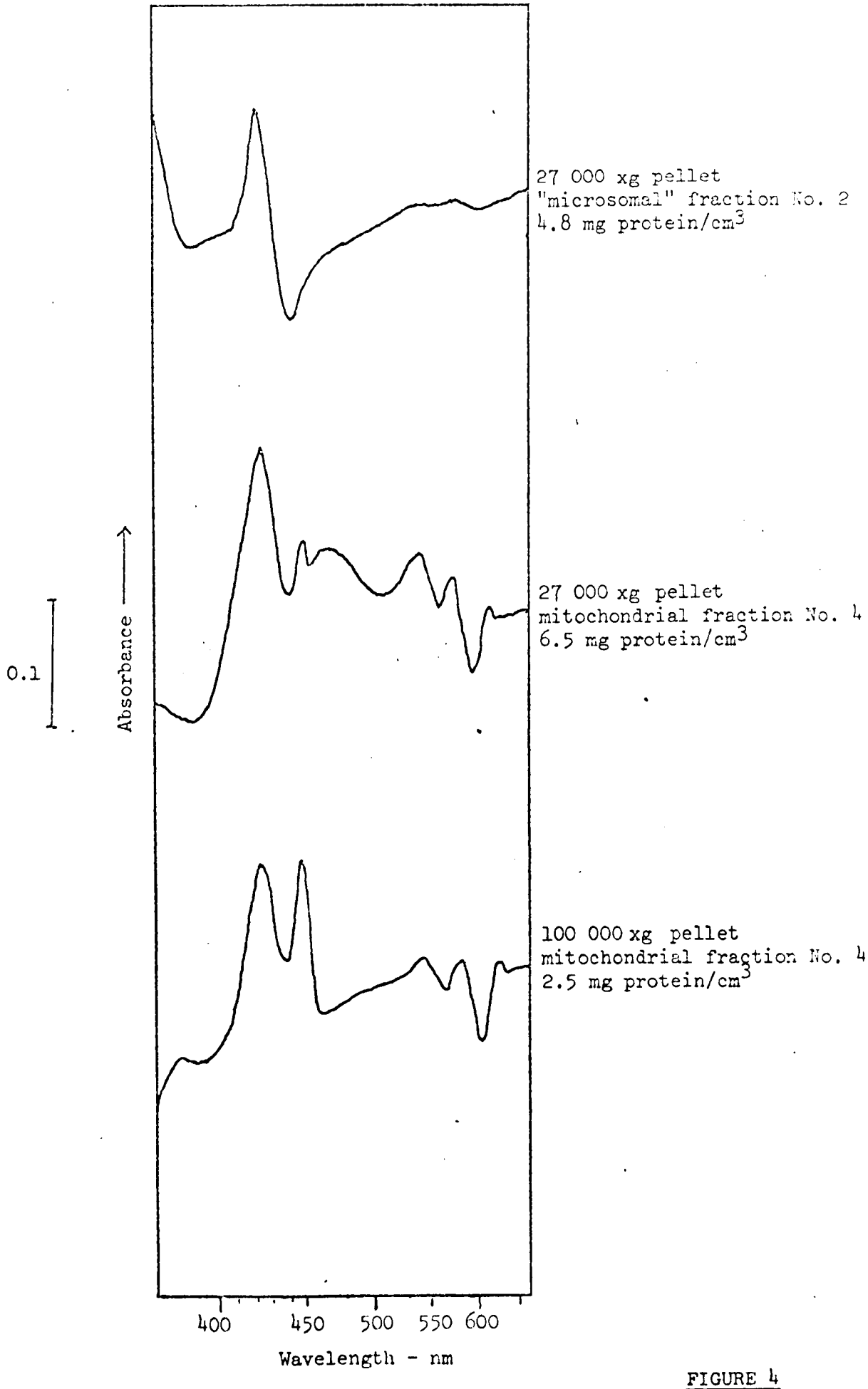


FIGURE 4

DIFFERENCE SPECTRA OF THE MITOCHONDRIAL FRACTION
FROM SUCROSE GRADIENT CENTRIFUGATION OF 100 000 xg PELLET

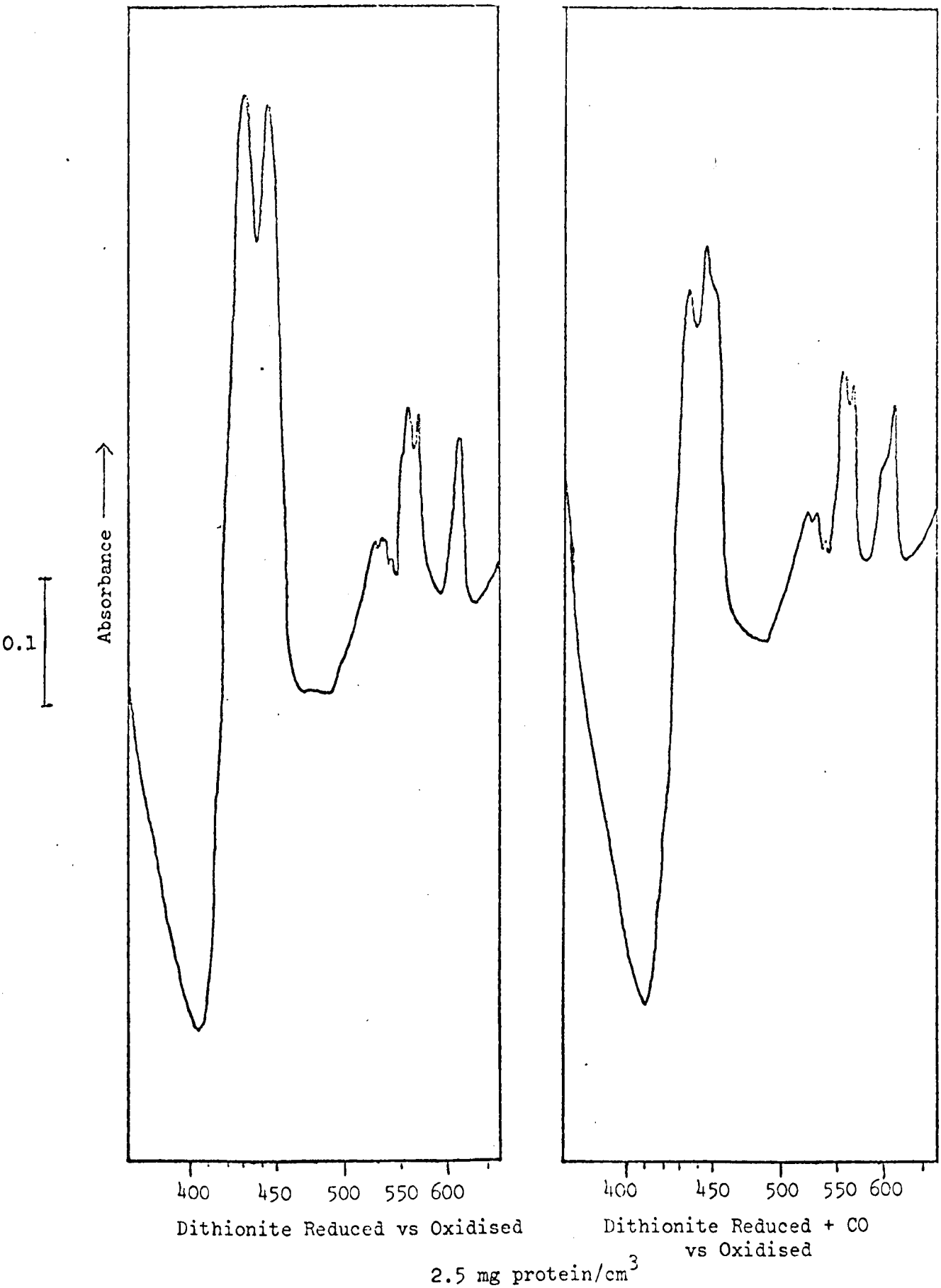


FIGURE 5